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Paolo Di Nardo · Sanjiv Dhingra
Dinender K. Singla *Editors*

Adult Stem Cells

Methods and Protocols

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Cover Caption: Immunofluoresce micrograph of alpha-sarcomeric actin (green) in differentiating cardiac progenitor cells grown on a biodegradable scaffold (phase contrast microscopy, Blue). Nuclei were stained with propidium iodide (red) (Felicia Carotenuto and Paolo Di Nardo)

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Preface

Degenerative diseases are the main cause of death globally and, owing to the lack of innovative drugs and several limits of organ transplantation, new cost-effective solutions for treating damaged organs are compelling needs. In this respect, the prowess of stem cells to repair tissues damaged by degenerative disorders has created universal interest, even if the related technologies are still in their infancy. Indeed, decades of intensive studies on stem cell behavior have not generated yet the clinical revolution so much announced and expected. This situation has been determined by many impeding factors, among which the lack of standardized protocols to isolate and handle stem cells has played a major role. Stem cell technology is among the most complex research endeavors and requires strict standardization of the materials and procedures, through a long-term process driven by merging the quantum of knowledge resident in different disciplines and international laboratories. This implies a strong impetus for change, in which cross-fertilization is encouraged to create a positive and explosive melting pot of ideas, methods, and expertise to benefit research and sustain advanced biomedical industries. Therefore, we should be aware that Stem Cell Standardization cannot be a concern of the biomedical field alone, but must involve the knowledge accumulated in a multiplicity of fields. The convergence between biomedicine and engineering holds promise to benefit both the patients and the industries. In this way only, we can hope to transform an artisanal activity, as stem cell research is today, in an industrialized process able to supply patients with efficient, safe, and cost-effective cell treatments.

This book collects stem cell protocols from some of the major laboratories involved in stem cell research in the world. They have made available their protocols in the hope to ignite a fruitful discussion on adult stem cells standardization to take further the first enthusiastic wave that has often produced only fragmented knowledge and unsuitable options for innovative treatments to be delivered in the clinical setting.

Inevitably, the book does not report protocols related to the isolation and culture of stem cells in all possible living tissues. The editors have selected some of those that they considered most reliable; it does not mean that the protocols excluded should be considered of minor interest.

Finally, the editors wish to express their deepest gratitude to Anna Maria Maccari for the invaluable support in collecting and helping to revise all manuscripts.

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Contents

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Chapter 1

A Simple Protocol to Isolate, Characterize, and Expand Dental Pulp Stem Cells

Federica Di Scipio, Andrea Elio Sprio, Maria Elisabetta Carere, Zhiqian Yang, and Giovanni Nicolao Berta

Abstract

Adult stem cells reside in body tissues to preserve organs and whole organism homeostasis. They are acquiring a prominent role in the contemporary medicine. Many protocols to isolate and cultivate adult stem cells have been so far described, though they are often lengthy, laborious, and require very expansive instruments, materials, and reagents. On this basis, we describe a simple, cheap but at the same time functional method to: (1) isolate dental pulp stem cells (DPSC), (2) expand and cultivate DPSC, (3) cryopreserve DPSC, (4) characterize DPSC, and (5) differentiate DPSC into both mesenchymal and non-mesenchymal lineages.

Key words Neural crest, Dental pulp stem cells, Cell extraction, Characterization, Expansion, Differentiation

1 Introduction

Stem cell (SC) research is one of the most interesting and promising areas of contemporary medicine. SC were postulated more than one century ago [1] and used for the first time in the clinics at the end of the 1960s [2, 3], thus paving the way for the current regenerative medicine era. From then, adult SC were retrieved in quite all body tissues, in which they reside to preserve organs and whole organism homeostasis. In the last two decades, in response to the increasing interest and request about artificial tissues, scientists have tried to cultivate resident cells, create engineered tissues, and design treatment for virtually every organ of the human body. In this Gold Rush, a multitude of adult stem cells have been isolated, characterized using a plethora of different and frequently generic "stemness" markers, and induced to differentiate toward several lineages. Nevertheless, the described features were frequently contradictory, depending on the anatomical site from which SC were extracted as well as on methods and equipment employed.

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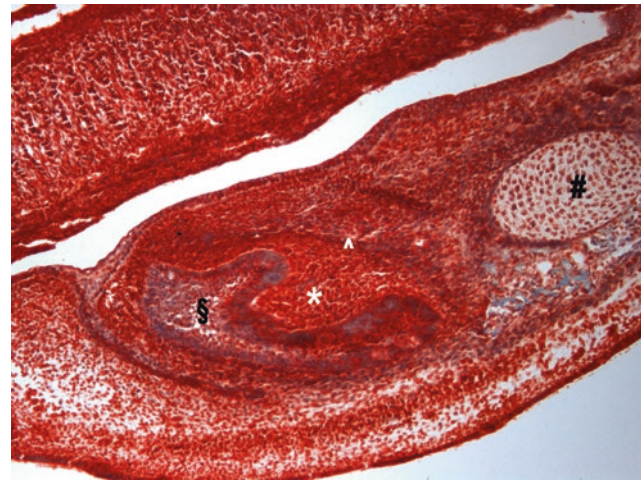

Fig. 1 Tricromical stained picture of a rat head at the 14 days of intrauterine life. Note the differences between the already differentiated Meckel's cartilage (#), from which the lower jawbone derives, and the dental papilla (*), entrapped between the enamel organ (§) and a fibrous connective membrane (^). In *blue*, mineralization confirms the status of differentiated tissues

In this scenario, the dental pulp could represent an important SC source. In fact, it is an ontogenetically derivative of "*the only interesting thing about vertebrates*" [4], the neural crest. This is a transient migrating cell population arising during the neural tube formation and able to differentiate into an impressive amount of different histotypes (e.g., neurons and glia of autonomous nervous system, pigment cells, many component of the splanchnocranium, and some portions of the heart) [5, 6].

In particular, during the odontogenesis, dental pulp derives from an ectomesenchymalization process in which the first pharyngeal arch epithelium interacts with the neural crest as mesenchymal counterpart. Moreover, these cells are early compartmentalized within the dental follicle and thus isolated and protected from massive differentiation stimuli that drive the jawbone development in the adjacent tissues (Fig. 1)[7].

Based on these evidences, it does not surprise that despite closely sharing the antigenic phenotype of bone marrow stromal cells, the dental pulp stem cells (DPSC) have exhibited a higher proliferation rate and demonstrated to differentiate in-vitro into bone, cartilage, adipose, neuronal, and cardiac precursors [8]. Nevertheless, they are so far underestimated to a certain extent, being up to now predominantly evaluated and employed for in vivo dental/calcifying tissue recovery.

Although in some mammals (e.g., rodents) incisive teeth grow throughout life inasmuch as open-rooted [9], the human third

molar tooth results equally singular. Its germ starts maturation around the 6th year of life. Until this time, embryonic tissues of dental lamina remain quiescent and undifferentiated within the jaw. Although crown mineralization begins at approximately 8th year of life, often third molar roots are incomplete at the age of eighteen. Thus, the structure of wisdom teeth still remains immature at the full age preserving a pool of undifferentiated cells [10].

Finally, despite consisting of a small amount of tissue, if compared to other stem cell sources, dental pulp retains the dual advantage of a richer content in stem cells and a less harmful/invasive procedure for their collection [11]. The easiness in isolation and high expansion potential in-vitro have made dental pulp stem cells very promising as a model system [12–14]. Here, we provide a simple, cheap, and reliable method to extract, isolate, and propagate adult stem cells from the dental pulp of both humans and animal models, without the employment of cell feeder or expensive medium supplements.

2 Materials

1. Phosphate buffered saline solution, pH 7.4.
2. Dulbecco's Phosphate Buffered Saline Modified, without calcium chloride and magnesium chloride.
3. Penicillin G (200 U/mL), Gentamicin sulphate (80 mg/mL) and 5 mg/mL Amphotericin B.
4. Collagenase/Dispase solution: 3 mg/mL collagenase type I and 4 mg/mL dispase II.
5. RPMI-1640 medium.
6. Fetal calf serum.
7. Trypsin/EDTA solution.
8. Dimethyl sulfoxide.
9. Paraformaldehyde.
10. Toluidine Blue solution.
11. Trypan Blue solution.
12. TRI Reagent solution (Sigma-Aldrich, Saint Louis, USA).
13. Chloroform.
14. Isopropyl alcohol.
15. Ethanol absolute, 99.8 %.
16. Deoxyribonuclease I kit (Fermentas International, Inc., Burlington, Canada).
17. RevertAid RT Reverse Transcription Kit (Fermentas International).

18. 10× PCR Buffer II (Roche Applied Science, Indianapolis, USA).
19. 25 mM $MgCl_2$ (Fermentas International).
20. 10 mM dNTP (Fermentas International).
21. Specific 10 mM primer (Sigma-Genosys).
22. Taq DNA Polymerase, recombinant (Fermentas International).
23. Triton X-100.
24. Fluoroshield Mounting Medium With DAPI (Abcam, Cambridge, UK).
25. Bovine serum albumin.
26. Sodium azide (NaN_3).
27. Facs buffer, PBS, 0.5–1 % BSA or 5–10 % FCS, 0.1 % NaN_3.
28. Adipogenic inductive medium; RPMI 1640 supplemented with 10 % (v/v) FCS, 1.7 mM insulin, 1 mM dexamethasone, and 0.5 mM methylisobutylxanthine, 100 U/mL penicillin G, 40 mg/mL gentamicin sulfate, and 2.5 mg/mL amphotericin B (Sigma-Aldrich).
29. Nile red staining solution.
30. Fluorescence Mounting Medium.
31. Osteogenic inductive medium; RPMI 1640 supplemented with 10 % (v/v) FCS, 10 mM b-glycerophosphate, 0.05 mM ascorbic acid, and 100 nM dexamethasone, 100 U/mL penicillin G, 40 mg/mL gentamicin sulfate, and 2.5 mg/mL amphotericin B (Sigma-Aldrich).
32. Alkaline phosphatase staining kit (BioOptica Milano SpA, Milano, Italy).
33. Chondrogenic inductive medium; RPMI 1640 supplemented with 10 % (v/v) FCS, 10 ng/mL TGF-β3, 0.025 mM ascorbic acid, and 100 nM dexamethasone, 100 U/mL penicillin G, 40 mg/mL gentamicin sulfate, and 2.5 mg/mL amphotericin B (Sigma-Aldrich).
34. β-Mercaptoethanol.
35. Retinoic acid.
36. Neuroglial inductive medium; RPMI 1640 supplemented with 10 % (v/v) FCS and 5 ng/mL platelet-derived growth factor, 10 ng/mL basic fibroblast growth factor, 252 ng/mL glial growth factor, and 14 mM forskolin (Sigma-Aldrich).
37. Antiboby against Neuron growth-associated protein 43 (GAP43) (Santa Cruz Biotechnology, Santa Cruz, CA).
38. Antiboby against Glial fibrillary acidic protein (GFAP) (DakoCytomation, Dako Italia SpA, Milano, Italy).
39. Antiboby against S100 protein (Rabbit) (Sigma-Aldrich).

40. Normal goat serum.
41. TRITC-labeled secondary.
42. CY3-labeled secondary antibody.
43. Alexa Fluor 488-labeled secondary antibody (Invitrogen).

3 Methods

3.1 Cell Isolation from Dental Pulp

Extracted tooth is collected, cleaned off blood and debris by means of a brush and a periodontal scaler, placed in PBS (pH 7.4) containing double strength penicillin G (200 U/mL), gentamicin sulfate (80 mg/mL), and 5 mg/mL amphotericin B and stored at 4 °C for up to overnight.

1. At the moment of sample processing, tooth is coronal graved with a dental bur. Particularly, after separating periodontal ligaments and root apical papilla from the root surface, dental pulp can be obtained from the cemento enamel junction cut by an odontomy to exhibit the pulp chamber (*see* **Note 1**).
2. From this step onward, aseptic techniques are required: under hood, use sterile tweezers to extract the pulp from the exposed pulpar chamber (*see* **Note 2**).
3. The pulp is laid out on a 100 mm petri dish (*see* **Note 3**) and chopped with a scalpel. Transfer into Eppendorf tube, wash thrice adding 1 mL sterile PBS and centrifugating at 1,000 rpm (~150 rcf) in a bench-top centrifuge for 5 min at 4 °C. After the third wash, eliminate supernatant and digest pellet with 1 mL collagenase (3 mg/mL)/dispase (4 mg/mL) solution for 1 h in a 37 °C water bath. Vortex every 15 min to help tissue disruption.
4. When the pulp is digested, wash thrice with 1 mL sterile PBS with centrifugation at 1,000 rpm (~150 rcf) for 5 min at 4 °C. Then, after eliminating the supernatant of the last centrifugation, resuspend the pellet with 1 mL RPMI-1640 medium, 100 U/mL penicillin G, 40 mg/mL gentamicin sulfate, and 2.5 mg/mL amphotericin B. To remove clumps and debris, pass cell suspension through a 70 μm strainer.
5. Add 3 mL RPMI-1640 medium and 1 mL fetal calf serum (FCS) and transfer into a 25-cm^2 flask (*see* **Note 4**).

3.2 Cell Expansion and Culture Conditions

1. Twenty-four hours later, nonadherent cells are removed, whereas the remaining cells are washed with sterile PBS and cultured in RPMI-1640 supplemented with 10 % FCS at 37 °C in a humidified 5 % CO_2 atmosphere.
2. After about 2 weeks in culture, non-stem cells go into senescence. The selection of the clones is possible by transferring the cells

remained adherent in 24-well plates and subsequently in 96-wells plates after serial dilution to reach the single cell/well density. Only wells enclosing homogeneous population are considered for further expansion (*see* **Note 5**).

3. Cell expansion is done by growing cells into 75-cm^2 flasks without any feeder layer or specific culture media. On reaching 80–90 % confluence, eliminate the exhausted medium, wash cells with PBS, add 3 mL of trypsin/EDTA solution (0.25 % trypsin, 0.02 % EDTA in PBS without calcium and magnesium), and incubate flasks at 37 °C for 3 min. When cells are detached, enzymatic action is inactivated by serum addition. Wash the obtained cell suspension in PBS with centrifugation at 1,000 rpm (~150 rcf) for 5 min and subcultivate cells at a 1:4 split ratio.

3.3 Cryopreservation

1. A cell pellet is obtained from a culture of expanded cells by using a trypsin/EDTA solution as described above.
2. Resuspend gently the pellet in 900 μL FCS and 100 μL dimethyl sulfoxide (DMSO) (*see* **Note 6**) at a concentration of 3–5 × 10^6 cells/mL in an ice/waterbath slurry. This 1 mL cell suspension is dispensed into cryovial and freezed at −20 °C for 2–3 h and then at −80 °C overnight before transferring the cells into liquid nitrogen (*see* **Note 7**).

3.4 Cell Characterization

To characterize cell volume and morphological complexity, flow cytometric analysis can be used.

3.4.1 Morphological Analysis of DPSC

1. Adherent, expanded DPSC are washed twice with PBS and detached by enzymatic digestion as described above. Wash once the single cell suspension at the density of 5 × 10^5 cells/mL in PBS with centrifugation at 1,000 rpm (~150 rcf) for 5 min.
2. Resuspend DPSC in PBS and measure directly the forward scatter and side scatter to analyze cell size and granularity. The DPSC should have a homogeneous morphological complexity (SSC, Side-SCatter) and dimension (FSC, Forward-SCatter).

3.4.2 Growth Characteristics

To evaluate the growth characteristics of DPSC, the plating efficiency and the population doubling time (Td) can be measured.

1. DPSC are seeded in 100-mm Petri dishes at the density of 2, 5, and 10 cells/cm^2 in medium supplemented with 10 % FCS. Cultures are set up in triplicate and incubated at 37 °C in 5 % CO_2 as usual.
2. After 2 weeks, wash the adherent cells with PBS and fix them with 4 % paraformaldehyde (PAF) for 15 min, stain with 0.1 % toluidine blue for 20 min, rinse with tap water and dry air.

3. Count macroscopic colonies with >30 cells using a phase contrast microscope. The plating efficiency is calculated as:

$$\frac{N_{\text{colonies}}}{N_{\text{plated-cells}}} \times 100$$

4. Regarding the T_d, to determine the growth rate, 1×10^5 DPSC are seeded into 25 cm^2 flasks with 5 mL of complete growth medium. The triplicate plates are counted every 24 h and viable cells are recognized through trypan blue exclusion staining. Briefly, mix cell suspension 1:1 with 0.4 % trypan blue solution in PBS. After at least 2 min of incubation, the cells in the trypan blue solution are loaded directly into a hemocytometer: nonviable cells are stained dark blue (*see* **Note 8**). Cell viability is calculated as the number of unstained (viable) cells divided by the total number of cells and expressed as a percentage. Given two measurements of a growing quantity, q_1 at time t_1 and q_2 at time t_2, T_d of cell lines is calculated as previously described [15]:

$$T_d = (t_2 - t_1) \times \left[\frac{\log(2)}{\log\left(\frac{q_2}{q_1}\right)} \right]$$

3.4.3 Stemness Marker Analysis

One important purpose of the characterization of stem cell cultures is tracking the presence of multi-pluripotency markers. Indeed, the confirmation of the stemness state should guarantee acknowledgment and expansion of the right cell type. According to the literature, DPSC should express such typical mesenchymal markers as CD105, CD73, and CD90, and lack expression of such specific blood lineage commitment markers as CD45, CD34. Moreover, they should express pluripotency markers including Oct4, Nanog, c-Myc, Sox2, stage-specific embryonic antigens (SSEA1, SSEA-3, SSEA-4) [12]. The presence of some stemness markers can be evaluated at m-RNA and/or protein levels and can be performed by means of RT-PCR reaction in the first case, and immunocytochemistry or FACS analyses in the last one. The three methods complete each other and can offer a full scenario about the extracted cells.

1. **mRNA expression analysis**
   (a) Eliminate growth media from cell culture and add 1 mL TRI Reagent in the culture dish per 10 cm^2 of culture dish surface area.
   (b) Lyse the cells in the culture dish by pipetting them up and down some times, move the lysate in a centrifuge tube,

and incubate for 5 min at room temperature to allow a total dissociation of the nucleoprotein complex.

(c) Put in 0.2 mL of chloroform per 1 mL of TRI Reagent used for homogenization. After closing the tube firmly, shake it energetically for some seconds. Incubate for 2–3 min at room temperature.

(d) Centrifuge the sample at 12,000 rpm (~13,400 rcf) for 15 min at 4 °C. The mixture divides into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA is in the aqueous phase that is approximately 50 % of the total volume.

(e) Take away the aqueous phase by tilting the tube at 45° and pipetting the solution out (*see* **Note 9**). Place the aqueous phase into a new tube.

(f) Add 0.5 mL of 100 % isopropanol to the aqueous phase, per 1 mL of TRI Reagent utilized for homogenization and leave at room temperature for 10 min.

(g) Centrifuge at 12,000 rpm (~13,400 rcf) for 10 min at 4° C, eliminate the supernatant leaving only the RNA pellet.

(h) Wash the pellet with 1 mL of 75 % ethanol per 1 mL of TRI Reagent used (*see* **Note 10**). Vortex the sample shortly, then centrifuge the tube at 7,500 rpm (~5,200 rcf) for 5 min at 4 °C. Eliminate the supernatant and air dry the RNA pellet for 5–10 min (*see* **Note 11**).

(i) Resuspend the RNA pellet in RNase-free water (20–50 μL) by pipetting the solution up and down some times and quantify at 260/280 nm.

(j) Add to an RNase-free, 0.5 mL microcentrifuge tube on ice: 1 μg RNA sample, 1 μL 10× DNase I Reaction Buffer, 1 μL DNase I (1 U/μL), and DEPC-treated water to 10 μL. Incubate at 37 °C for 30 min.

(k) Add 1 μL 25 mM EDTA and incubate at 65 °C for 10 min.

(l) Use RNA as a template for reverse transcriptase. Place 1 μL random examers (0.2 μg/μL), 1 μL 10 mM dNTP Mix, 4 μL 5× Reaction Buffer, 2 μL 0.1 M DTT, and 1 μL RevertAid Reverse Transcriptase (200 U) to have a total volume of 20 μL. Incubate for 10 min at 25 °C, then 60 min at 42 °C, and finish the reaction by heating at 70 °C for 10 min.

(m) Add to an RNase-free, 0.5-mL microcentrifuge tube on ice: 16 μL DEPC-treated water, 2.5 μL 10× PCR Buffer II, 0.5 μL10 mM dNTP Mix, 1.5 μL 25 mM $MgCl_2$, 0.75 μL 10 μM Primer Forward, 0.75 μL 10 μM Primer Reverse, 0.5 μL TAQ, and 2.5 μL cDNA. Amplificate the cDNA at the idoneous annealing temperature and cycle counts.

(n) Separate amplification products by means of agarose gel electrophoresis. Stain DNA bands with ethidium bromide, digitally capture and analyze them using software packages.

2. **Immunocytochemistry analysis**

(a) For the immunostaining, cells on the slides are fixed with 4 % PAF for 15 min at room temperature.

(b) After washing three times in PBS, block cells with 10 % serum in PBS triton 0.1 % for 1 h at room temperature (see Note 12). Subsequently, incubate with primary antibodies in PBS overnight at 4 °C.

(c) After washing in PBS three times for 5 min each, add the secondary antibodies in PBS for 1 h at room temperature in the dark. Wash the slides three times for 5 min each with PBS in the dark and add a cover slip along with fluorescence mounting medium containing the nuclear stain, DAPI. Slides can be visualized under a fluorescence microscope.

3. **Flow cytometric analysis**

(a) Harvest and wash the DPSCs as described above.

(b) Resuspend up to 1×10^7cells (*see* **Note 13**) in ice cold facs buffer (PBS, 0.5–1 % BSA or 5–10 % FCS, 0.1 % NaN_3) (*see* **Note 14**).

(c) Add 100 μL of cell suspension to each polystyrene round-bottom tube.

(d) Add 100 μL of blocking antibody to each sample (blocking antibody diluted in buffer at 1:50 ratio). Incubate on ice for 20 min. Centrifuge at 1,500 rpm (~200 rcf) for 5 min at 4 °C and remove supernatant.

(e) Add 0.1–10 μg/mL of the primary antibody and incubate for at least 30 min at 4 °C in the dark. Wash the cells thrice in buffer centrifuging at 1,500 rpm (~200 rcf) for 5 min. Resuspend in at least 200 μL of ice cold buffer.

(f) Dilute the secondary antibody in the buffer according to the manufacturer's instructions, resuspend cells in this solution, and incubate for 30 min at 4 °C in the dark. Wash the cells thrice by centrifugation at 1,500 rpm (~200 rcf) for 5 min and resuspend in at least 200 μL of ice cold buffer.

(g) Analyze cell suspensions on the flow cytometer as soon as possible (see **Note 15**).

3.5 Differentiation Potential of DPSC In Vitro

The main fate of DPSC consists in their differentiation into dental cells, but they are able to differentiate into both mesenchymal and non-mesenchymal lineages in vitro. This property is recognized as a specific characteristic of these cells. However, according to the International Society for Cellular Therapy, for being defined as

mesenchymal stem cells, they must be able to differentiate in vitro at least into three cellular lineages: adipocytes, osteoblasts, and chondrocytes [16]. In addition, DPSC are also able to differentiate into neural precursors in vitro.

1. **Adipogenic differentiation**
   (a) Seed 1×10^5 in vitro expanded DPSC on triplicate 22 mm square coverslips in a 6-well plate in RPMI-1640 containing 10 % FCS at 37 °C in 5 % CO_2 as usual.
   (b) After cell adhesion, remove the culture media and add an equal volume of adipogenic induction media consisting of RPMI-1640 containing 10 % FCS, 1.7 mM insulin, 1 mM dexamethasone, and 0.5 mM methylisobutylxanthine. Every 2 days, change the adipogenic induction medium (*see* **Note 16**).
   (c) After 5 days of induction, remove the media and gently rinse the adipogenic culture twice with PBS.
   (d) Fix the adipogenic culture in the 6-well plate with 4 % PAF for 15 min at room temperature.
   (e) Wash thrice with PBS, incubate with 200 nM nile red staining solution for 10 min at room temperature protecting from light. Wash again with distilled water until excess nile red stain is removed, mount with fluorescence mounting medium on glass slides. This dye will emphasize lipid vesicles occurrence visible by fluorescent microscope (excitation 515–560 nm).
2. **Osteogenic differentiation**
   (a) Seed 1×10^5 in vitro expanded DPSC on triplicate 22 mm square coverslips in a 6-well plate in RPMI-1640 containing 10 % FCS at 37 °C in 5 % CO_2 as usual.
   (b) After cell adhesion, remove the culture media and add an equal volume of osteogenic induction media consisting of RPMI-1640 containing 10 % FCS, supplemented with 10 mM b-glycerophosphate, 0.05 mM ascorbic acid, and 100 nM dexamethasone. Replace the osteogenic medium twice a week for 3 weeks.
   (c) After 3 weeks of induction, remove the media and gently rinse the osteogenic culture twice with PBS. Fix with 4 % PAF for 15 min at room temperature, and then rinse three times with distilled H_2O.
   (d) Evaluate osteogenic differentiation by means of an alkaline phosphatase staining kit. Alkaline phosphatase is an important marker for osteoblast activity. Positive alkaline phosphatase enzymatic activity is visualized as a black deposits on a yellow background.

3. **Chondrogenic differentiation**
   (a) Seed 1×10^5 in vitro expanded DPSC on triplicate 22 mm square coverslips in a 6-well plate in RPMI-1640 containing 10 % FCS at 37 °C in 5 % CO_2 as usual.
   (b) After cell adhesion, remove the culture media and add an equal volume of chondrogenic induction media consisting of RPMI-1640 containing 10 % fetal calf serum, supplemented with 10 ng/mL TGF-β3, 0.025 mM ascorbic acid, and 100 nM dexamethasone. Replace the chondrogenic medium twice a week for three weeks.
   (c) After three weeks, differentiated cells are highlighted with toluidine blue staining as above described. If compared with untreated DPSC, round-shaped cell clusters characterized by a basophilic cytoplasm can be visualized.
4. **Neuroglial differentiation**
   (a) Seed 1×10^5 in vitro expanded DPSC on triplicate 22 mm square coverslips in a 6-well plate in RPMI-1640 containing 10 % FCS at 37 °C in 5 % CO_2 as usual.
   (b) After cell adhesion, remove the culture media and pretreat cells with 1 mM b-mercaptoethanol in a complete medium. After 24 h, wash and add new complete medium supplemented with 35 ng/mL retinoic acid for 72 h.
   (c) Following this four-day pretreatment, it is possible to begin the neuroglial induction. After two washings, DPSC are grown in a complete medium supplemented with 5 ng/mL platelet-derived growth factor, 10 ng/mL basic fibroblast growth factor, 252 ng/mL glial growth factor, and 14 mM forskolin for 2 weeks.
   (d) It is possible to confirm the induction of the neuroglial phenotype by specific immunostaining, e.g. neuron growth-associated protein 43 (GAP43), glial fibrillary acidic protein (GFAP), and S100 protein (S100).
   (e) Antigen detection can be achieved by means of specie-specific labeled secondary antibodies (e.g. Alexa 488, TRITC, and CY3). Add a cover slip along with fluorescence mounting medium containing the nuclear stain, DAPI. Slides can be visualized under a fluorescence microscope (Alexa 488: excitation/emission maxima of 495/519 nm; TRITC: excitation/emission maxima of 557/576 nm; CY3: excitation/emission maxima of 554/568).

4 Notes

1. Tooth coronal incision must be performed intermittently preserving the tooth surface wet with cold water to avoid overheating of the pulp.

2. It is also possible to remove the pulp by using a barbed broach.
3. Not to dry the pulp: sprinkle it with PBS.
4. To promote cell adhesion, use medium with 20 % FCS.
5. Examination of the adherent cells by microscope reveals them growing from single foci, or colonies, called "colony-forming unit" (CFU).
6. Final medium composition is 10 % DMSO/90 % FCS v/v.
7. It is important that the cell freezing occurs slowly to prevent the formation of crystals that can damage cells.
8. Live cells with intact cell membranes are not stained by trypan blue dye, instead the dead ones are permeable and take up the color.
9. When removing the aqueous phase, be careful to not take any of the interphase or organic layer into the tip.
10. The RNA can be preserved in 75 % ethanol at least 1 year at −20 °C, or 1 week at 4 °C.
11. Do not allow the RNA to dry entirely, because the pellet can lose solubility. Partly dissolved RNA samples have an A260/280 ratio <1.6.
12. The incubation with the blocking serum is necessary to suppress the non-specific binding with the antibody. The blocking serum ideally should be derived from the same species in which the secondary antibody is produced.
13. It is always useful to check the viability of the cells, which should be around 95 % but not less than 90 %.
14. It is important to use ice cold solutions, since low temperature and presence of sodium azide prevent the modulation and internalization of surface antigens that can produce a loss of fluorescence intensity.
15. For long-term storage (>16 h), after **step 6**, add to the cell suspension 1–4 % PAF, incubate for 15 min at room temperature, centrifuge at 1,500 rpm (~200 rcf) for 5 min, and resuspend cells in at least 200 μL of ice cold PBS. Fixation avoids deterioration and stabilize the light scatter.
16. Every differentiation must have the negative control represented by DPSC cultivated for the same time of the related differentiation but with the normal medium.

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Chapter 2

Derivation of Mesenchymal Stem Cells from Embryonic Stem Cells: A Non-Variable and Inexhaustive Source of Adult Stem Cells

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Abstract

Mesenchymal stem cells (MSCs) provide an opportunity to bring the field of regenerative medicine to realization. A lot of clinical trials are presently trying to establish their applicability in real-world scenarios. Some of the biggest challenges encountered in bringing MSCs from bench to bedside are the number of MSCs required, their procurement from various sources, and the batch-to-batch variability. This often leads to inconclusive results within and between different studies. Therefore, we have hereby proposed a simple protocol to source mesenchymal stem cells through differentiation of embryonic stem cells.

Key words Mesenchymal stem cells, Embryonic stem cells, Differentiation, Regenerative medicine, Batch variation, Non-Variable MSC source

1 Introduction

Embryonic stem cells (ESCs) are in-vitro-established isolated inner cell mass from the blastocyst stage of the embryo. They are known to differentiate into all the cell types including cells from all the three germ layers. Further, they have been proved to be capable of long-term proliferation, while retaining their telomerase activity and karyotype stability [1]. Their differentiation capabilities are also conserved, leading to the possibility of continuous source of required cell types specific to each patient over many years. When ESCs are differentiated to terminal cells like skin cells, cardiomyocytes, and retinal cells, there are suspicions of these cells not properly integrating into the tissues. To remedy such situations, it is believed the approach of ESC differentiation to adult stem cells like mesenchymal stem cells (MSCs) would be more apt for regenerative medicine [2]. These ESC-derived MSCs have been known to display similar properties to MSCs sourced from adult

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individuals, like possible differentiation into the cells of the target tissues, immune-privilege, immunomodulation, and activation of resident stem cells through paracrine actions [3, 4].

Transplantation of MSCs into damaged tissue has been proven to help with reemergence of healthy cells leading to betterment of the affected tissues [5]. These properties make MSCs a promising candidate for regenerative medicine, allowing for a wide variety of clinical applications. Currently, numerous clinical trials are being carried out to validate the applicability of MSCs. Their safety has been proved consistent through all the studies, with varying efficacies [6].

The conventional methodologies of extracting MSCs from individuals though widely being applied present a lot of hurdles. As the donors age, the quality of MSCs and its regenerative ability deteriorate [7]. It has also been widely reported, cryopreserved MSCs have not been found to be very efficient for transplantation [8]. Though MSCs can be sourced from various tissues like bone marrow, adipose tissue, and dental pulp, the nature of procurement is highly invasive in nature [9]. Autologous sources of MSCs have been found to be too cumbersome and limited in their availability, allogeneic MSCs do provide a better option [10]. Even then, allogeneic sources need to be properly matched to the patient [11].

Here, we have described a method for differentiation of mouse embryonic stem cells (mESCs) to MSCs to help establish a regular source of mouse MSCs with the aim of avoiding the problems associated with procuring new fresh animals, batch-to-batch variations, and multiple sources. The outlined directions will provide a step-by-step approach for the regular maintenance of mESCs and their subsequent differentiation into MSCs using specific medium and detailed procedures. Thereupon an easy protocol for the successive identification, isolation, and purification of the MSCs is outlined, which is carried out through immunostaining and passaging. Further maintenance and expansion of the mouse mesenchymal stem cells is elaborated using appropriate characterization techniques like immunostaining. Attention has been given to the employment of regular consumables and medium components found in the general laboratory environment to enable easy setup for beginners.

2 Material

2.1 Cells

1. Mouse embryonic fibroblasts (commercially available).
2. Mouse embryonic stem cells (commercially available).

2.2 Supplies and Equipment

1. 15 mL and 50 mL conical centrifuge tubes.
2. 1.5 mL centrifuge tubes.

3. 6 cm tissue-culture grade plates.
4. Tissue culture flasks.
5. Cell counter and specific slides.
6. Glass slides and cover slips.
7. Pipettes and pipette tips.
8. Sterile waste aspiration system.
9. Sterile 10 cm non-tissue culture petridishes.
10. Inverted microscope.
11. Fluorescence microscope.
12. Macroscope.
13. Centrifuge with multiple rotors, buckets, and holders.
14. CO_2 incubators.
15. −80 °C freezers.
16. Liquid nitrogen freezer.

2.3 Reagents and Buffers (See Note 1)

1. Phosphate Buffered Saline (PBS), Calcium/Magnesium free.
2. Dulbecco's Modified Eagle Medium—high glucose with L-glutamine and sodium pyruvate (DMEM-hg).
3. Dulbecco's Modified Eagle Medium—low glucose (DMEM-lg).
4. 0.5 % Trypsin EDTA/TrypLE™ Express.
5. Fetal Bovine Serum (FBS, heat-inactivated).
6. Embryonic stem cell-qualified FBS (ES-FBS).
7. Dimethyl Sulfoxide (DMSO).
8. 70 % Ethanol.
9. Penicillin/Streptomycin (PenStrep) 100×.
10. Non-Essential Amino Acids (NEAA) 100×.
11. L-Glutamine 100×.
12. β-Mercaptoethanol, 55 mM.
13. 0.1 % gelatin.
14. Leukemia Inhibitory Factory (LIF).
15. Tryphan blue.
16. Primary and secondary antibodies (*see* **Note 2**).
17. 3 % paraformaldehyde (PFA).
18. Mitomycin C.
19. Bovine serum albumin solution (BSA) (*see* **Note 3**).
20. Antibody Dilution Buffer (1× PBS/1 % BSA/0.3 % Triton ×100).

2.4 Media Solutions (If Required Filter Through 0.2 μm Filters)

1. MEF medium: DMEM hg, 1× NEAA, 10 μL/mL from 55 mM (stock) β-mercaptoethanol, 1× penstrep, 10 % FBS (*see* **Note 4**).
2. MMC medium (mitotic inactivation): MEF medium with 10 μg/mL of final concentration of Mitomycin C (*see* **Note 5**).
3. mESC medium: DMEM hg, 1× NEAA, 10 μL/mL from 55 mM (stock) β-mercaptoethanol, 15 % ES FBS, 10 ng/mL of LIF (*see* **Notes 4** and **5**).
4. MSC medium: DMEM-lg, 15 % FBS, 1× PenStrep, 10 μL/mL from 55 mM (stock) β-mercaptoethanol (*see* **Note 4**).
5. MSC differentiation (Diff) medium: mESC medium without LIF (*see* **Note 4**).
6. Freezing medium (MEF): 90 % FBS, 10 % DMSO.
7. Freezing medium (mESC): 50 % mESC medium, 40 % ES-FBS, 10 % DMSO.
8. Freezing medium (MSC): 50 % MSC medium, 40 % ES-FBS, 10 % DMSO.

3 Methods

3.1 MEF Maintenance and Inactivation

1. Thaw MEFs in warm MEF medium. Pellet the cells at 200 × *g* for 7 min. Discard supernatant. Resuspend in warm MEF medium. Plate onto tissue culture ware of desire.
2. Replace medium daily.
3. MEFs take approximately 4–5 days to reach confluency of 95 % when plated at 10 %.
4. Passage of the MEFs can be carried out by first giving three washes with PBS followed by Trypsin treatment for 1–3 min (*see* **Note 6**). Once the cells detach, collect in 15 mL tube, deactivate trypsin by adding warm MEF medium. Pellet the cells at 200 × *g* for 7 min. Resuspend in warm MEF medium. Plate onto tissue culture ware of desire (*see* **Note 7**).
5. The mitotic inactivation of the MEFs is carried out by replacing its medium with MMC medium. Place it in an incubator for 2 h. Then wash with PBS three times and follow with trypsin treatment. Once pelleted, either freeze in MEF freezing medium for later use or plate onto plates. The MMC-treated MEFs are hereon referred to as feeders.
6. Prior to plating feeders, coat the plates with 0.1 % gelatin for at least 1–2 h. Plate feeders at 30,000–50,000 cells per cm^2.

3.2 mESC Maintenance

1. 1 day after plating feeders, replace the medium with mESC medium 1 h prior to plating mESC.

2. mESC need to be thawed in mESC medium. Pellet the cells at 200 × *g* for 7 min. Discard supernatant. Resuspend in warm mESC medium. Seed them on the feeder plates.
3. Replace the medium regularly.
4. mESC grow as dome-shaped colonies (*see* Fig. 1a). They need to be passaged every 3–4 days.
5. Passaging is carried out as for MEFs. Three washes with PBS followed by Trypsin treatment for 1–3 min (*see* **Note 6**). Once the cells detach, collect in 15 mL tube, deactivate trypsin by adding warm MEF medium. Pellet the cells at 200 × *g* for 7 min. Resuspend in warm mESC medium. Seed on new feeder plates at 3000–5000 cells per 6 cm plate.

3.3 Differentiation (See Fig. 2)

1. Progress as passaging (*see* **Note 8**).
2. Count the cells. Resuspend at 700–900 cells/30–50 μL of Diff medium (*see* **Note 9**).
3. Place approx. 30–50 μL on the lid of sterile 10 cm non-tissue culture petridishes as shown in Fig. 1b. Place the lid on the plate. The cells now will be present in hanging drops. Place in an incubator for 3 days. These cells will form round ball like structures called embryoid bodies (EBs) (*see* Fig. 1c).
4. On day 3 collect the EBs and plate onto gelatin-coated plates using Diff medium.

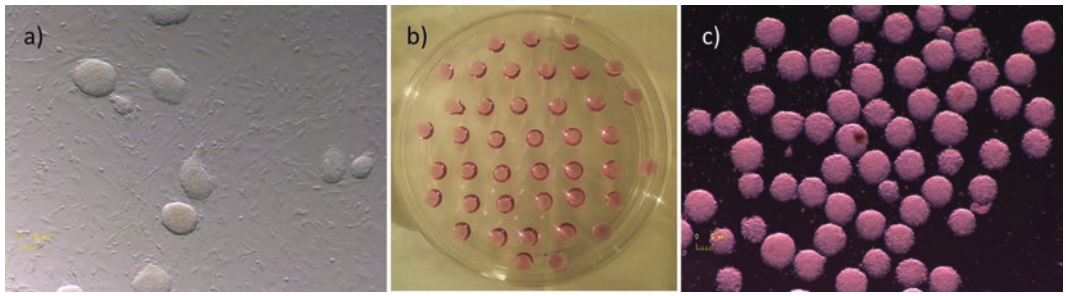

Fig. 1 (**a**) Dome-shaped mESC colonies on a bed of feeders, (**b**) 40 μL hanging drops with mESCs on the lid of a sterile non-tissue-culture petridish, (**c**) Floating embryoid bodies

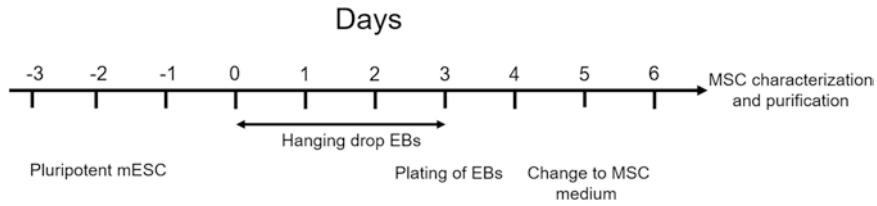

Fig. 2 Schematic timeline of the MSC differentiation from mESC

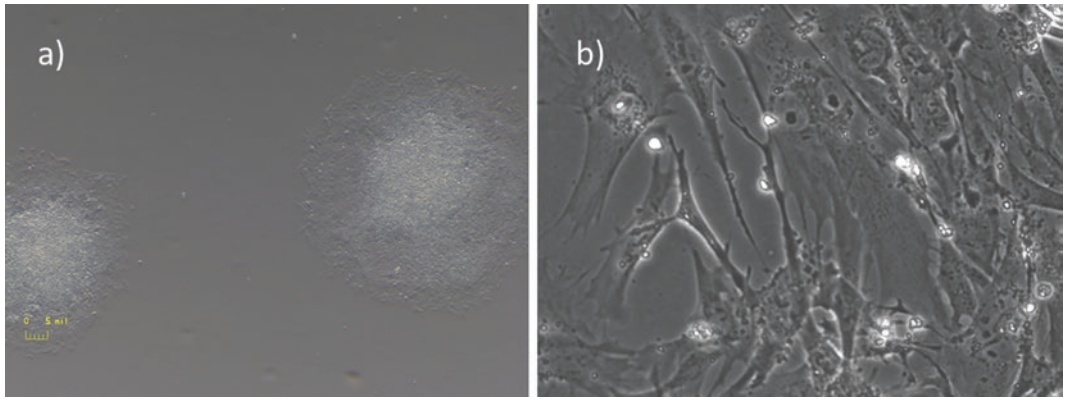

Fig. 3 (**a**) Flattened embryoid bodies, (**b**) MSC-like cells at 40×

5. On day 4–5 (once the EBs have mostly flattened) (*see* Fig. 3a), replace the medium of EBs with MSC medium (*see* **Note 10**).
6. Observe for MSC like morphology (*see* Fig. 3b).

3.4 Enriching MSCs

1. Passaging can be carried out once the EBs have completely flattened and many cell types have shown death.

   Steps 2–6 *allow MSC enrichment based on its strong plastic-adherence property.*
2. Trypsin treatment needs to be done in stages.
3. Stage 1 involves trypsin treatment for 1 min. A quick rinse with PBS to wash away non-MSC cell types.
4. At the end, mostly MSCs and fibroblasts will be left behind.
5. In stage 2 reintroduce trypsin, for 2–3 min. This will help in detaching the required MSCs.
6. After plating the passaged cells in a new dish, allow the cells 1–2 h of attachment time. MSCs tend to attach quickly. Afterward, very gently, wash the dish surface with MSC medium and discard the wash.
7. These steps will successively allow a greater number of MSCs to populate the plate.

3.5 Immunostaining of ESC and MSCs

1. General Antibodies for mESC—Oct4a, SOX2, SSEA1.
2. General Antibodies for MSC—CD 44 (+), CD 29 (+), and CD 34 (−).
3. Grow the cells on cover slips (*see* **Note 11**).
4. Fix the cells in 3 % PFA for 15 min.
5. Block the cells in desired blocking buffer for 60 min.
6. While blocking, prepare primary antibody by appropriate dilution in antibody dilution buffer.

7. Aspirate blocking solution, apply diluted primary antibody.
8. Incubate overnight at 4 °C.
9. Rinse three times in 1× PBS for 5 min each.
10. Incubate specimen in secondary antibody diluted in Antibody Dilution Buffer for 1–2 h. at room temperature in the dark.
11. Rinse three times in 1× PBS for 5 min each.
12. Mount and observe under fluorescence microscope (*see* Figs. 4 and 5).

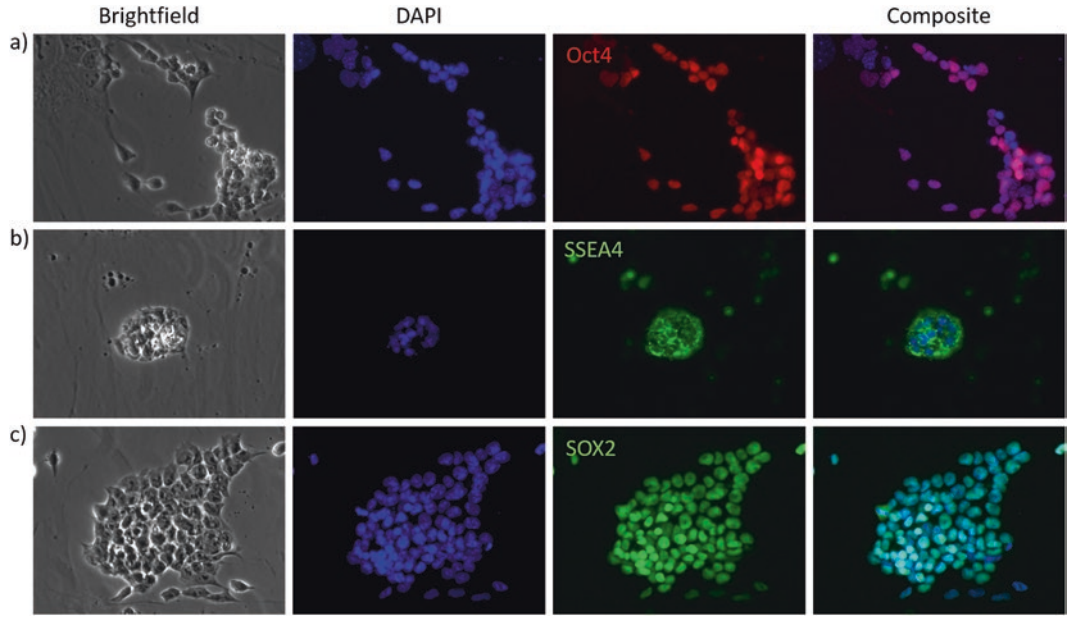

Fig. 4 Immunofluorescence staining of mESCs at 40×; (**a**) Oct4, (**b**) SSEA4, (**c**) SOX2

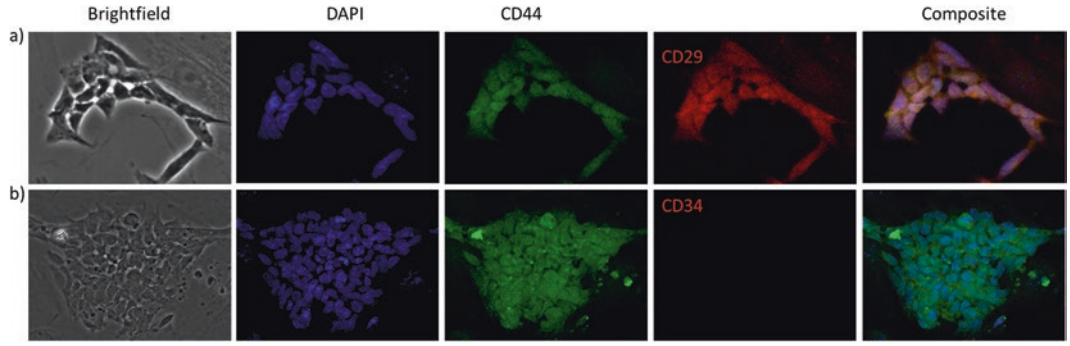

Fig. 5 Immunofluorescence staining of MSCs at 40×; (**a**) CD44 (*Green*) & CD29 (*Red*), (**b**) CD44(*Green*) & CD34 (*Red*)

4 Notes

1. It is better to avail most of these commercially to simplify quality assessment and avoiding batch variability.
2. There are a lot of antibodies for pluripotency markers and MSCs. One need not procure all, but the number to be tested should depend on the nature of downstream analyses.
3. 1 % BSA. Serum specific to secondary antibodies for blocking has been reported to be better.
4. β-Mercaptoethanol ethanol needs to be added freshly.
5. Mitomycin C is soluble in warm water or DMSO. Premade mitomycin C solution is commercially available.
6. 1× PenStrep may be added if necessary.
7. Longer trypsin will decrease the quality of the MEFs and attachment of mESCs.
8. For freezing the MEFs, once the cells are pelleted, resuspend the cells in 5 mL medium. Count the cells. Re-pellet at 200 g for 7 min. Now suspend in MEF freezing medium at 1–5 million cells/mL. Store at −80°C or in liquid nitrogen.
9. Rather than adding trypsin to the complete plate, individual colonies can be picked up under the macroscope. This is done to ensure fewer amounts of MEFs being picked up (MEFs will interfere in proper embryoid formation). Also, the colonies with the best round dome-shape can be picked up to ensure highest pluripotent stem cells, which would not later compromise differentiation (*see* Fig. 1a).
10. This number of mESC in the hanging drop needs to be optimized depending on the mESC cell line that the lab has procured.
11. If death of EBs and cells is observed extensively, or the eventual yield of MSCs is less, start adding Diff medium:MSC medium at 50:50 from day 4 to day 6. Then change over to complete MSC medium.
12. Any other desired format may be used, like chamber slides, glass bottomed dishes, etc.
13. Once immunostaining confirms presence of MSCs, the cells may also be sorted through FACS system.

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Chapter 3

Compact Bone-Derived Multipotent Mesenchymal Stromal Cells (MSCs) for the Treatment of Sjogren's-like Disease in NOD Mice

Ghada Abu Elghanam, Younan Liu, Saeed Khalili, Dongdong Fang, and Simon D. Tran

Abstract

Compact bone (cortical or dense bone) is among the organs that contain multipotent mesenchymal stromal cells (MSCs). Unlike bone marrow plugs where MSCs were initially isolated, compact bone has minimal (amount of) hematopoietic cells and thus facilitates the MSCs isolation process. In vitro, MSCs from compact bone show multipotency and differentiation into mesenchymal tissues such as bone, adipose, and cartilage, under certain conditions. MSCs therapy has been promising in preclinical and clinical studies against autoimmune diseases. Not only can MSCs replace the lost tissue through their regenerative properties, but they can also control the autoimmune attacks by immunoregulatory cytokines. This protocol describes the use of compact bone-derived MSCs to preserve salivary function (saliva flow/output) in the NOD (nonobese diabetic) mouse model affected with Sjogren's-like disease.

Key words Compact bone, Mesenchymal stromal cells, MSCs, Autoimmune diseases, Sjogren's syndrome, Sjogren's-like disease, Saliva, Hyposalivation, Xerostomia, Nonobese Diabetic (NOD) mouse

1 Introduction

Saliva is a secretory fluid that protects the oral tissues and aids in swallowing; thus its loss is problematic. Sjogren's syndrome (SS), radiotherapy to the head and neck area for cancer treatment, and the intake of some drugs (such as antihypertensive agents) can all cause saliva loss (salivary hypofunction, xerostomia). SS is a common chronic autoimmune disease characterized by focal lymphocytes infiltration into the salivary and lacrimal glands leading to xerostomia and exophthalmia [1]. Women are affected nine times more than men and are mostly postmenopausal. SS can be restricted to the secretory glands only, primary SS, or coexists with other autoimmune diseases like rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE), secondary SS. The etiology of SS is

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believed to be multifactorial; certain environmental factors seem to provoke an existing genetic predisposition [2].

Current treatments of SS are mostly palliative; no curative treatment exists until now. Therapeutic options include artificial saliva and tears, parasympathomimetic agents like pilocarpine (Salagen) to overstimulate the residual functioning salivary tissue. In more severe cases with serious systemic involvement, immunosuppressant drugs like methotrexate might be used [3]. Unfortunately, all previously mentioned treatments cannot restore the damaged cells nor enhance the formation of new ones. This has encouraged several research attempts to find a suitable treatment for SS via stem cells therapy or tissue engineering [4–6].

MSCs have been used to treat autoimmune diseases [7–10] due to their immunomodulatory and anti-inflammatory properties that can alleviate and attenuate the immune attack against the organs [11, 12]. In addition, these cells have a powerful regenerative capacity that will support the existing tissue and probably trigger the formation of new cells. Differentiation of MSCs into local tissue cells type is possible if these cells are from a mesenchymal origin while the formation of new cells in the non-mesenchymal tissue is mainly the responsibility of the local progenitor cells. In this chapter, we describe a protocol that uses mesenchymal stromal cells from compact bone to preserve the salivary flow rate in non-obese diabetic mice (NOD). NOD mouse is a commonly used animal model to study SS [13–15, 10, 16]. These mice show salivary and lacrimal lymphocytic infiltration and subsequent secretion loss similar to SS human patients. Recent studies have reported that MSCs from NOD bone marrow (BM) have a very low expression of the surface marker CXCR4 which is responsible for homing BM stem cells to the inflammation site [17]. Transplantation of normal MSCs from healthy donors to NOD mice will eliminate this obstacle. The newly introduced cells can be trafficked to the inflammation site and arrest the attack against the glandular cells with anti-inflammatory and immunomodulatory properties. This chapter will demonstrate the steps for isolating compact bone MSCs and their enrichment, to the final injection into the NOD mice.

2 Materials

2.1 Preparing an Aseptic Condition

It is important to maintain the sterility throughout this procedure. Once the animal (donor) is euthanized, all procedures are carried out in the laminar flow hood. All surgical equipments and tools should be sterilized prior to their use. All surfaces, gloves, and any other items introduced into the laminar flow hood should be sprayed generously with 70% ethanol.

2.2 Animals

1. 6–8-weeks-old CByB6F1-eGFP male mice were used for transplantation experiments (male GFP transgenic mouse [C57BL/6TgH (ACTbEGFP)10sb/J (stock # 003291) were bred with female BALB/c (stock # 00651)].
2. Five animals were kept in the same cage and provided with standard animal care in the animal facility.

2.3 Animal Surgery

1. CO_2 chamber.
2. Sterilized dissecting straight scissors.
3. Sterilized straight tweezers.
4. Sterilized scalpel.
5. 50 mL conical centrifuge tube.
6. Washing buffer: 1 mL antibiotic-antimycotic, 2 mL fetal bovine serum (FBS), 97 mL PBS.

2.4 Isolation of Compact Bone $CD45^-/TER\text{-}119^-$ Multipotent Mesenchymal Stromal Cells

1. Sterilized straight tweezers.
2. Sterilized scalpel.
3. 70 mm ceramic mortar and pestle.
4. 70 μm nylon cell strainer.
5. 50 mL conical centrifuge tube.
6. MesenCult™ MSC Basal Medium (Mouse), (STEMCELL).
7. MesenCult™ Mesenchymal Stem Cell Stimulatory Supplements (Mouse), (STEMCELL).
8. MSCs growth medium: one bottle of MesenCult™ MSc Basal Medium, one bottle MesenCult™ Mesenchymal Stem Cell Stimulatory Supplements, 5 mL antibiotic-antimycotic, 5 mL L-Glutamine.
9. Collagenase I solution: 0.25 gm collagenase I, 80 mL PBS, 20 mL fetal bovine serum (FBS) then filter sterilize the solution.
10. Parafilm.
11. 60 and 100 mm cell culture dish.
12. 5 mL round-bottom polystyrene tube.
13. 3% acetic acid in methylene blue.
14. Glass cover slip 22 × 30 mm.
15. Hemocytometer.

2.5 Selection and Culture of Compact Bone $CD45^-/TER\text{-}119^-$ Multipotent Mesenchymal Stromal Cells

1. EasySep™, Mouse Mesenchymal Progenitor Enrichment Kit (STEMCELL).
2. EasySep™ Magnet (STEMCELL).
3. MSCs growth medium (*see* **item 8** in Subheading 2.4).
4. 100 mm cell culture dish.
5. 15 mL conical centrifuge tube.

6. 0.25% Trypsin–EDTA.
7. Trypan blue stain 0.4%.
8. Glass cover slip 22 × 30 mm.
9. Hemocytometer.

2.6 Osteogenic Differentiation

1. Osteoblast differentiation medium: α-MEM medium, 1% antibiotic-antimycotic (100 U/mL penicillin-G, 100 μg/mL streptomycin and 0.25 μg/mL Amphotericin B), supplemented with 10% FBS, 0.1 mM ascorbic acid, and 10^{-8} M dexamethasone, 2 mM β-glycerophosphate.
2. MSCs growth medium (*see* **item 8** in Subheading 2.4).
3. 6-well cell culture plate.
4. 5% silver nitrate solution in dH_2O.

2.7 Adipogenic Differentiation

1. Adipogenic differentiation medium: α-MEM medium, 1% antibiotic-antimycotic (100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL Amphotericin B), 10^{-8} M dexamethasone, 10 μg/mL insulin, 0.5 mM 1-Methyl-3-Isobutylxanthine 0.5 μM hydrocortisone, 60 μM Indomethacin.
2. MSCs growth medium (*see* **item 8** in Subheading 2.4).
3. 6-well cell culture plate.
4. Oil red O stain: 0.3% oil red O staining solution. 0.3 g oil red O stain dissolved in 100 mL of 60% isopropanol.
5. 10% Neutral Buffered Formalin.

2.8 Chondrogenic Differentiation

1. StemXVivo™ Chondrogenic Base Media (R&D Systems).
2. StemXVivo™ Human/Mouse Chondrogenic Supplement 100× (R&D Systems).
3. Chondrogenic differentiation medium: 0.5 mL StemXVivo Chondrogenic Base Media, 5 μL StemXVivo Human/Mouse Chondrogenic Supplement with, 5 μL antibiotic-antimycotic (optional).
4. Anti-Collagen II antibody (Abcam).
5. 15 mL conical centrifuge tube.

2.9 Flow Cytometry Analysis

1. Anti-Mouse TER-119 APC, clone: TER-119 (eBioscience).
2. Anti-Mouse CD11b, clone: M1/70 (eBioscience).
3. Anti-Mouse CD106 (VCAM-1) eFlour® 450, clone: 429 (eBioscience).
4. Anti-Mouse CD105 eFlour® 450, clone: MJ7/18, (eBioscience).
5. Anti-Mouse Ly-6A/E (Sca-1), clone: D7 (eBioscience).

6. Anti-Mouse CD73 PE-Cyanine7, clone: eBioTY/11.8 (TY/11.8), (eBioscience).
7. Anti-Mouse/Rat CD29 PE-Cyanine7 (Integrin beta 1), clone: eBioHMb1–1(HMb1–1) (eBioscience).
8. Anti-Mouse CD44 PE-Cyanine7, clone: IM7 (eBioscience).
9. Anti-Mouse CD45, clone: 30-F11 (BD Bioscience).
10. BD LSRFortessa cell analyzer.
11. Flowjo software.

2.10 Colony Forming Unit-Fibroblast (CFU-F) Assay

1. 6-well cell culture plate.
2. MSCs growth medium (*see* **item 8** in Subheading 2.4).
3. Giemsa stain (Sigma).

2.11 Cell Transplantation

1. MSCs growth medium (*see* **item 8** in Subheading 2.4).
2. 0.25% Trypsin–EDTA.
3. Normal saline.
4. 0.5 mL Insulin syringe.
5. 1.5 mL sterilized Eppendorf tube.
6. Mouse restrainer.
7. Alcohol swap.
8. Red heat lamp.

3 Methods

3.1 Animal Surgery

This section describes the steps for the surgical dissection of the animal, removing muscle tissue from the limbs and preparing the long bone for $CD45^-/TER\text{-}119^-$ cells isolation.

1. Euthanize the animal using the accepted method at your facility.
2. Place the body on the supine position and disinfect it generously with 70% ethanol.
3. Use a pair of dissecting straight scissors and straight tweezers to cut the fur and the skin from the base of the neck toward the pelvic region. From the midline incision made, cut the skin toward the wrist region and the ankle region respectively. Remove the skin that covers the limbs completely.
4. Remove as much as possible of the attached muscles and the ligaments with the aid of dissecting straight scissors and a scalpel. Avoid cutting the bone at this stage.
5. Cut the leg/forearm just below the ankle/wrist joint and the femur/humerus just above the hip/shoulder joint.

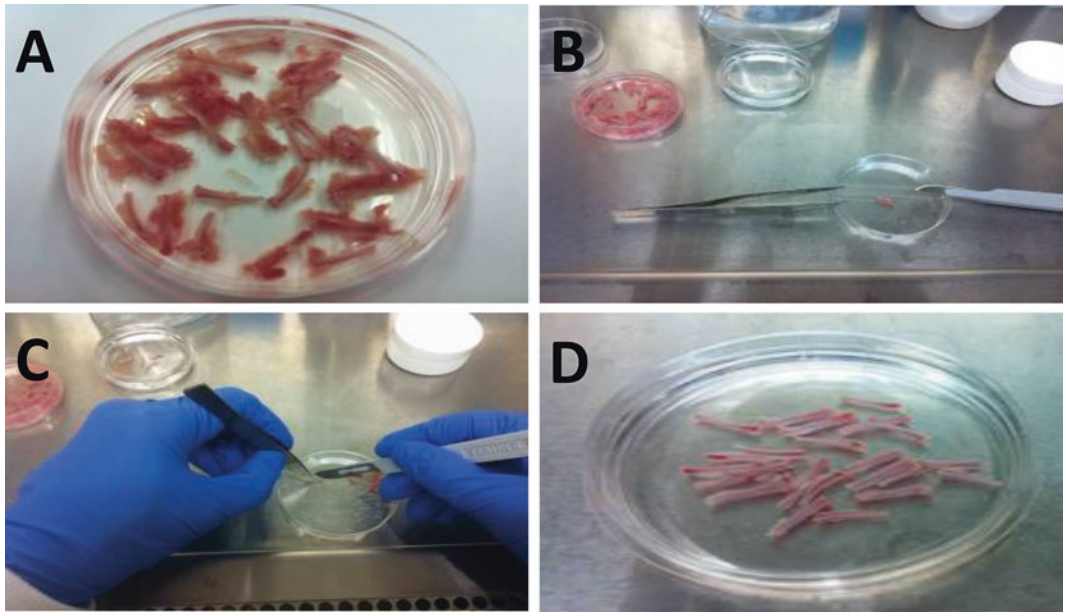

Fig. 1 Surgical resection of long bones and removal of any surrounding soft tissues. (**a**) Long bones after resection from the body of the mouse. (**b**) Instrumentations setup for the complete removal of soft tissues still attached to the long bones. (**c**) Removal of the soft tissues with the aid of straight tweezers and a scalpel. (**d**) Long bones denuded of soft tissues were immersed in washing buffer

6. Transfer the limbs to a new 100 mm cell culture dish containing the washing buffer (*see* Fig. 1a).
7. Remove any remaining muscles or ligaments and then separate the bones (*see* Fig. 1b, c, d).
8. Transfer the bones to a 50 mL conical centrifuge tube containing the washing buffer, and gently shake the tube. Change the buffer three times (*see* Fig. 1).

3.2 Isolation of Compact Bone CD45⁻/TER-119⁻ Multipotent Mesenchymal Stromal Cells

This section describes the steps for the isolation of compact bone cells from five mice by collagenase I digestion and outgrowth from cultured bone fragments.

1. All solutions used must be at room temperature.
2. Sterilize the mortar and pestle with 70% ethanol and allow it to dry in the laminar flow hood before the beginning of the cells isolation.
3. Place the bones in the sterilized mortar (*see* Fig. 2a).
4. Gently crack the bones with the pestle and then break them down into smaller pieces (*see* **Note 1**).
5. Add 10 mL of the washing buffer to the bone fragments and then pipette the solution up and down several times (*see* **Note 2**). Discard the buffer and add another 10 mL, repeat

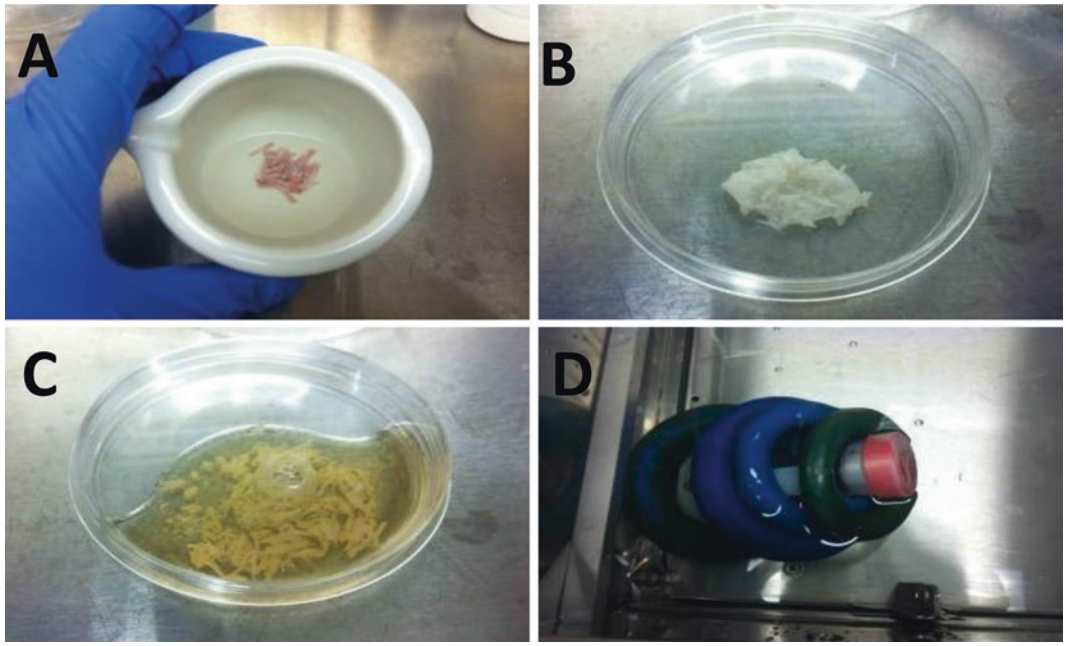

Fig. 2 Bone marrow separation and isolation of mesenchymal stromal cells from the compact bone. (**a**) Long bones in a sterilized mortar. (**b**) The marrow-free bone fragments in a 100 mm cell culture dish. (**c**) Collagenase I solution with bone fragments in 100 mm cell culture dish. (**d**) Incubation of bone fragments with collagenase I in a 37 °C shaker water bath

pipetting up to six times or until the bone fragments turn white in color. At this step, the depletion of bone marrow cells is reached.

6. Place the bone fragments in a 100 mm cell culture (*see* Fig. 2b).
7. Add enough collagenase I solution (5–7 mL) to cover all the bone fragments (*see* Fig. 2c).
8. Leave the collagenase I solution for 3–5 min 37 °C humid incubator; cut the softened bone fragments with the aid of straight tweezers and a scalpel into 1–2 mm. Higher cell yield will be obtained with smaller fragments.
9. Add more collagenase I solution (8–10 mL) to the fragments to make the final volume 15 mL and then transfer them to a new 50 mL conical centrifuge tube (*see* **Note 3**).
10. Seal the tube with parafilm and place it in a 37 °C shaker bath for 45 min (*see* Fig. 2d).
11. Stop the enzymatic digestion by adding 20 mL washing buffer, making the final volume 35 mL.
12. Transfer the supernatant to a new 50 mL conical centrifuge tube and wash the bone fragments with an additional 10 mL of washing buffer.

13. Aspirate and add the buffer to the supernatant from previous step and filter them through a 70 μm nylon cell strainer.
14. Count the cells with 3% acetic acid in methylene blue (*see* **Note 4**).
15. Centrifuge at 300 × *g* for 10 min.
16. Aspirate the supernatant and resuspend the cells in MSCs growth medium. Seed cells at 3–5 × 10^5 cells/cm^2 in a 100 mm cell culture dish. Culture the cells in 37 °C humid incubator with 5% CO_2. Change the media every 2–3 days.
17. Transfer the bone fragments from the 50 mL conical centrifuge tube to a new 100 mm cell culture dish.
18. Add enough MSCs growth medium to cover the bone fragments.
19. Culture the bone fragments following the same procedure (*see* **step 16** in Subheading 3.2, **Note 5**, Fig. 3).
20. When the cells reach 70–80% confluency (*see* **Note 6**), detach the cells with 0.25%Trypsin–EDTA for 2–3 min in the 37 °C humid incubator or until the cells become rounded and lose attachment to the culture vessel.
21. Centrifuge the cells at 300 × *g* for 5 min.
22. Aspirate the supernatant and resuspend the cells in 5 mL of MSCs growth medium then perform cell counting.
23. Centrifuge the cells at 300 × *g* for 5 min.

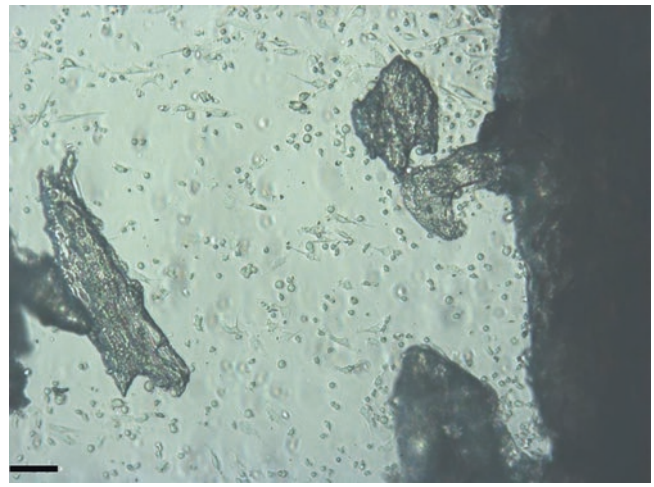

Fig. 3 Compact Bone cells by outgrowth method. Phase contrast photomicrograph showing the cell outgrowth from the edges of the compact bone fragments (shown as *black irregular objects*; for example on the right side of the photomicrograph). Scale bar = 38 μm

24. Aspirate part of the supernatant and leave a volume, according to your cell count to make the final concentration 2–5 × 10^7 cells/mL.
25. Transfer the cells to 5 mL polystyrene round-bottom tubes, which will fit later in the EasySep™ magnet.
26. Keep the cells on ice until the selection step.

3.3 Selection and Culture of Compact Bone CD45⁻/TER-119⁻ Multipotent Mesenchymal Stromal Cells

This section describes the enrichment of **CD45⁻/TER-119⁻** MSCs by using a cocktail of biotinylated antibodies designed against non-MSCs (CD45⁺, TER-119⁺ cells) while the MSCs are left unlabeled.

1. Isolation of the CD45⁻/TER-119⁻ cells was performed using EasySep™ mouse mesenchymal progenitor enrichment kit (STEMCELL).
2. Use the cells from the previous step (*see* **step 26** in Subheading 3.2).
3. Add the mouse mesenchymal progenitor enrichment cocktail to the cell suspension at a ratio of 50 μL/mL, mix well, and incubate at 4 °C refrigerator for 15 min.
4. Add 4 mL of MesenCult MSc Basal Medium and centrifuge the suspension at 400 × *g* for 5 min.
5. Aspirate the supernatant and add fresh medium to suspend the cells at 2–5 × 10^7 cells/mL.
6. Add 250 μL of Biotin selection cocktail to every 1 mL of cells, mix well, and incubate in 4 °C refrigerator for 15 min.
7. Vortex the M Prog™ Magnetic Microparticles for 30 seconds or until no visible clumps inside the tube and then add 150 μL to each 1 mL of the cells suspension. Mix well and incubate in 4 °C refrigerator for 15 min.
8. Add more medium to the suspension to bring the final volume to 2.5 mL and then pipette up and down gently for two to three times.
9. Place the tube uncapped in the EasySep™ magnet for 5 min in laminar flow hood (*see* **Note 7**).
10. Invert the EasySep™ magnet with the tube inside and pour the solution containing the target cells into a new conical centrifuge tube in one motion (*see* **Note 8**).
11. Add more MSCs growth medium to the cell suspension and then perform cell counting.
12. Plate cells at 3–5 × 10^5cells/cm^2 in 100 mm cell culture dish (*see* **Note 9**).
13. Passage cells at 70–80% confluency with 0.25% Trypsin–EDTA.

3.4 Multilineage Differentiation

This section describes the osteogenic, adipogenic and the chondrogenic differentiation procedures, and the final verification by the appropriate staining method.

3.4.1 Osteogenic Differentiation

1. Cells are seeded in a 6-well cell culture plate with MSCs growth medium and incubated in a 37 °C humid incubator until they reach approximately 50–70% confluency.
2. Aspirate the growth medium and replace with 2 mL of the osteogenic differentiation medium per well.
3. Incubate the cells in a 37 °C humid incubator with 5% CO_2 and change the medium every 2–3 days.
4. After 3 weeks, osteogenic differentiation is visualized by the Von Kossa staining method (*see* Fig. 4a).

3.4.2 Adipogenic Differentiation

1. Cells are seeded in a 6-well plate with MSCs growth medium and incubated in a 37 °C humid incubator with 5% CO_2 until they reach approximately 90–100% confluency. (It takes approximately 1–4 days).
2. Aspirate the growth medium and replace it with 2 mL of the adipogenic differentiation medium per well.

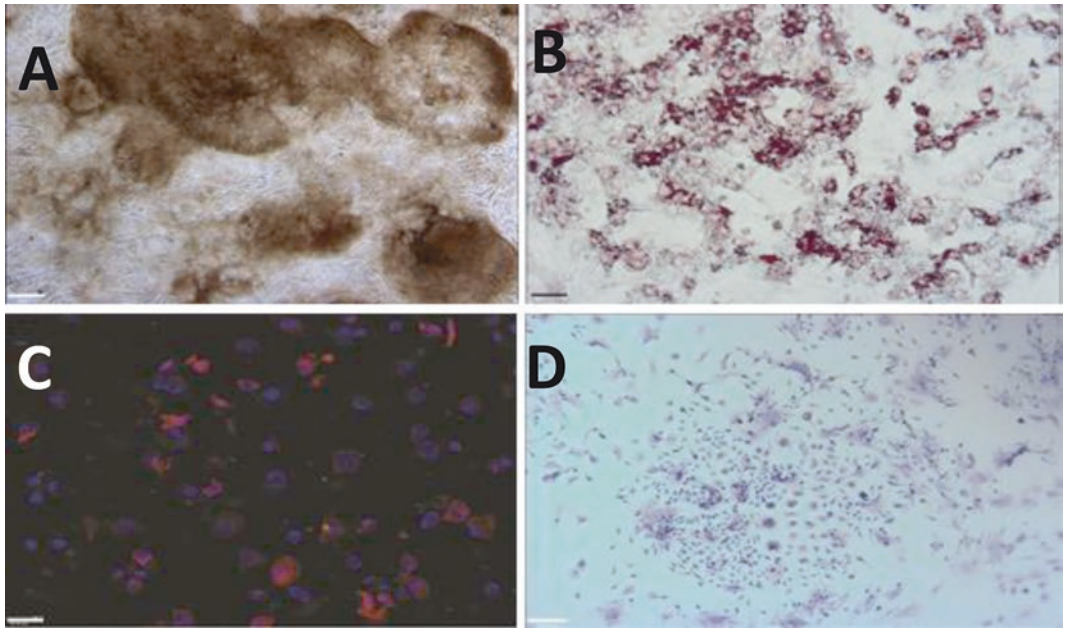

Fig. 4 Multilineage differentiation of compact bone and CFU formation. (**a**) Von Kossa staining for osteogenic differentiation. (**b**) Oil red O staining for Adipogenic Differentiation. (**c**) Collagen type II immunofluorescent staining for chondrogenic differentiation (from Ref. 16). (**d**) CFU formation stained with Giemsa stain. Scale bar = 38 μm

3. Incubate the cells in a 37 °C humid incubator with 5% CO_2 and change the medium every 2–3 days.
4. After 3 weeks of adipogenic differentiation is visualized by Oil Red O staining (*see* Fig. 4b).

3.4.3 Chondrogenic Differentiation

1. Transfer 250,000–500,000 cells to a 15 mL conical centrifuge tube and centrifuge at 300 × *g* for 5 min.
2. Aspirate the supernatant and add 5 mL pre-warmed StemXVivo™ Chondrogenic Base Media and centrifuge at 200 × *g* for 5 min.
3. Aspirate the supernatant and resuspend the cell pellet with 0.5 mL prewarmed chondrogenic differentiation medium and then gently pipette up and down two to three times.
4. Centrifuge the cells again at 200 × *g* for 5 min without discarding the medium. Allow the detachment of the cell pellet from the bottom of the tube without disturbing it. Loosen the cap of the tube to allow gas exchange and place it in a rack vertically inside the 37 °C humid incubator with 5% CO_2.
5. In 1–2 days the pellet will form a small ball that will remain the same size during the period of the differentiation.
6. Change the medium with freshly prepared chondrogenic differentiation medium each time every 2–3 days (*see* **Note 10**). Culture the cells for up to 21 days.
7. Cell pellet is retrieved, cryopreserved in OCT, and sectioned with a microtome at 7 μm thickness.
8. Chondrogenic differentiation is further confirmed with the immunofluorescence staining for Collagen II (*see* Fig. 4c).

3.5 Colony Forming Unit (CFU) Assay

1. Seed the cells (*see* **step 12** in Subheading 3.3) at 1 × 10^4 cells/cm^2 and culture them for 10–20 days.
2. Change medium every 2–3 days.
3. When the colonies are formed, the staining step can be started.
4. Remove the media and gently wash the cells twice with PBS.
5. Allow the cells to dry, and then add enough methanol to cover the cells, and incubate for 5 min at room temperature (around 25 °C) in a fume hood.
6. Remove the methanol and allow drying for 5 min.
7. Add a diluted Giemsa (1:20 in distilled water) and incubate for 5 min.
8. Remove the Giemsa stain and wash gently with dH_2O until the water is clear (*see* Fig. 4d).

3.6 Flow Cytometry

1. Harvest the cells at 70–80% confluency at passage 3 with 0.25% Trypsin–EDTA.
2. Cells are stained with the provided antibodies (*see* Subheading 2.9) and prepared for the sorting using BD LSRFortessa cell analyzer.
3. Data obtained from the analyzer was analyzed using Flowjo software (*see* Fig. 5).

3.7 Cell Transplantation

This section describes the steps for harvesting the cells and the final transplantation into the mice via tail vein.

1. Cells used for the transplantation are harvested shortly prior to the procedure.
2. Add enough 0.25% Trypsin–EDTA to a 70–80% confluent cell culture dish at passages 3–5, incubate in a 37 °C humid incubator with 5% CO_2 for 2–3 min or until the cells detach (*see* **Note 11**).
3. Deactivate the 0.25% Trypsin–EDTA by adding an equal volume of the MSCs growth medium.
4. Collect the cells and centrifuge at 300 × *g* for 5 min.
5. Aspirate the supernatant and wash the cells with normal saline three times and then centrifuge at 300 × *g* for 5 min after each wash.

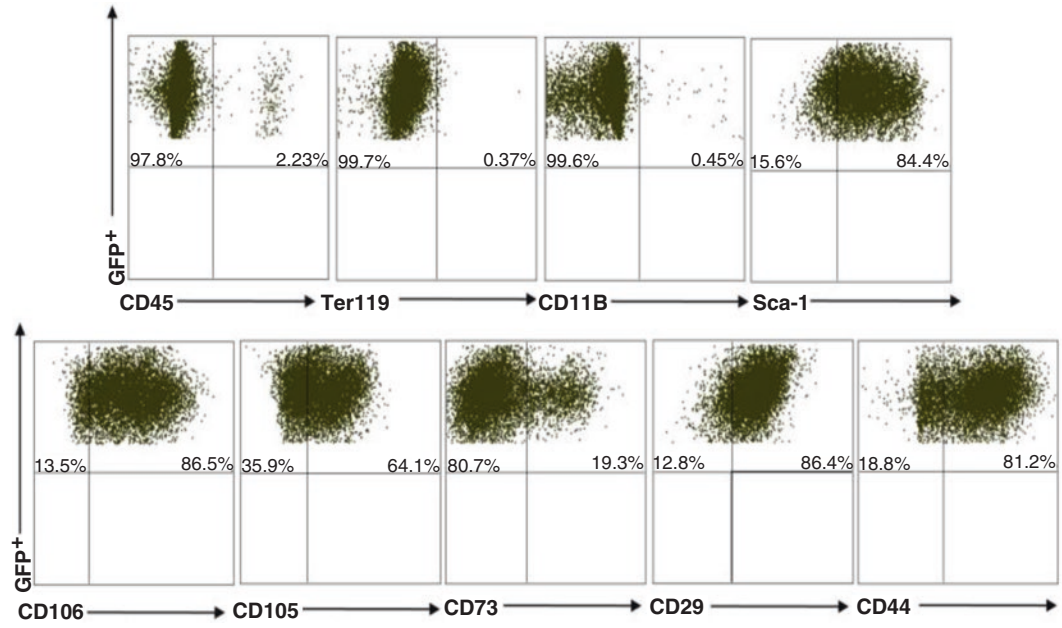

Fig. 5 FACS analysis of MACS purified CD45$^-$ /TER-119$^-$ cells from compact bone shows 97.8% CD45$^-$, 99.7% TER-119$^-$, 99.6% CD11b$^-$ and 84.4% Sca1$^+$, 86.5% CD106$^+$, 64.1% CD105$^+$, 19.3% CD73$^+$, 86.4% CD29$^+$, 81.2% CD44$^+$ cells (from Ref. 16)

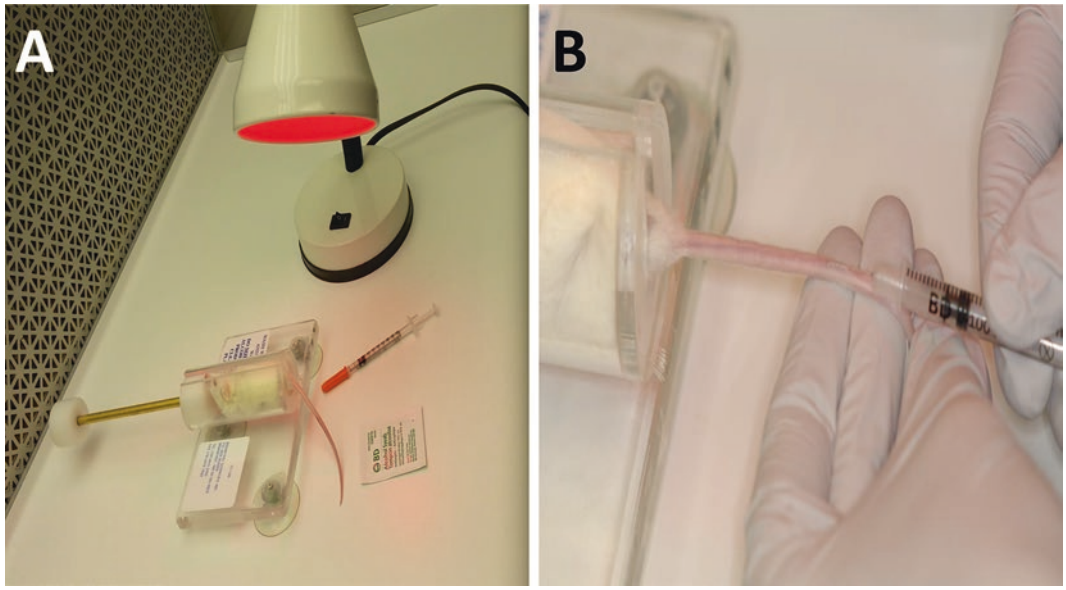

Fig. 6 Cell Transplantation. (**a**) The apparatus and the materials required for the cell transplantation. (**b**) The needle should be inserted parallel to the tail vein in the middle third

6. Suspend the cells in normal saline at a ratio of 1×10^7 cells/100 μL in a 1.5 mL eppendorf tube and keep it on ice until the injection time.
7. Warm up the cell suspension to body temperature by holding the eppendorf tube between your fingers and vortex it prior to the injection.
8. Using a 0.5 mL insulin syringe, aspirate 100 μL of the cell suspension for each mouse.
9. Place the mouse in the restrainer in sternal position (*see* **Note 12**, Fig. 6a).
10. Direct the red heat lamp at the lower two thirds of the mouse tail for 5–10 min or until the veins are visible and dilated.
11. Rub the tail with an alcohol swab and insert the syringe needle (the bevel facing upward) in the middle third section of the lateral tail vein, parallel to the tail. Inject 100 μL slowly at a constant speed (*see* **Note 13**, Fig. 6b).
12. Apply a gentle pressure at the injection site to stop the bleeding with an alcohol swab for 1–2 min.
13. Monitor the mouse after the injection for any adverse reactions.
14. Repeat the injection twice a week for 2 consecutive weeks.
15. For saliva collection, sedate the mouse with isoflurane and then inject 0.05 mg/kg pilocarpine subcutaneously. Collect whole

saliva by placing a micropipette (inside a 0.5 mL eppendorf tube) intraorally for 10 min from the start of pilocarpine injection. Saliva is collected every 4 weeks after the cell therapy.

4 Notes

1. Avoid trituration that may devitalize the cells.
2. This step is performed to remove the bone marrow from the bone fragments.
3. Bone from one mouse requires 3 mL of collagenase I solution.
4. Acetic acid with methylene blue is used to remove any remaining RBCs that may cause inaccuracy of the cells number.
5. When the bone fragments are cultured, it is advised not to move the plate for 3 days after the initial plating. At this stage, the cells are outgrowing from the bone surface toward the culture vessel. Any movement at the first 3 days might interfere with this process.
6. Cells from the bone fragments outgrowth are harvested after the removal of the bone fragments. The bigger fragments are suctioned individually using a 2 mL sterile pipette while the smaller ones are collected at one side of the cell culture dish by washing them with PBS and then suctioned altogether at once.
7. At this step, all the labeled cells that are basically the non-MSCs will move toward the magnet side. This will purify the free MSCs in the solution.
8. Avoid any tapping of the tube inside the magnet as you may dislodge the unwanted non-MSCs cells.
9. Selection is recommended after the first passage as the cell number is higher than the freshly isolated ones.
10. Avoid disturbing or losing the cell pellet during medium exchange.
11. Prepare cells instantly before the injection, as the cells tend to form clumps if harvested much earlier.
12. Selecting the appropriate size of the mouse restrainer/holder is an important factor. Smaller size restrainer can interfere with the mouse breathing and cause discomfort, while larger size allows mouse movements during injection and consequently you lose control when performing the injection.
13. Change to another injection site if the injection does not flow passively (e.g., high pressure on the piston of the syringe), as the injected cells most likely diffused into the surrounding tissue instead of inside the vein.

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Chapter 4

Manipulating the Proliferative Potential of Cardiomyocytes by Gene Transfer

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Abstract

In contrast to prenatal life, cardiomyocyte proliferation in mammals is rapidly blunted after birth; as a consequence, clinically significant cardiac regeneration does not occur in adulthood. Thus, the modulation of cardiomyocyte proliferation by gene transfer offers an invaluable opportunity to both understand the mechanisms regulating renewal of these cells in the fetus and identify novel strategies for myocardial repair.

In this Chapter, we report an exhaustive protocol to isolate, culture, and manipulate the properties of neonatal ventricular rat cardiomyocytes by small RNA transfection or transduction with viral vectors based on the adeno-associated virus, which exhibit exquisite tropism for these cells. We also provide techniques to assess DNA synthesis and cell proliferation.

Key words Cardiomyocytes, EdU, BrdU, Phospho-histone 3, Aurora B, MicroRNAs, AAV vectors

1 Introduction

There is a tremendous need to develop new therapeutic strategies to stimulate cardiac regeneration in patients with myocardial infarction and heart failure. According to the World Health Organization, over 20 million people are diagnosed every year with heart failure, which mostly ensues as a consequence of ischemic cardiomyopathy; of these, 80 % reside in low- and middle-income countries (http://www.who.int/cardiovascular_diseases/en/). Despite resynchronization therapy and use of medical-assisted devices, the prognosis of heart failure remains poor, with mortality estimated at 40 % only after 4 years from diagnosis [1].

A leading pathogenic mechanism underlying heart failure relates to the incapacity of the adult heart to generate a significant number of new cardiomyocytes during the adult life. Carbon dating of cardiomyocyte DNA indicates that the renewal capacity of the heart is less than 50 % in a 70-year lifetime [2], an estimate that is consistent with information obtained by imaging mass spectrometry, showing that cell renewal of cardiomyocytes is in the order of

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approximately 1 % every year [3]. Thus, an impelling need exists to develop innovative therapies able to stimulate cardiac regeneration as a mean to counteract heart failure by providing the heart with new contractile cells.

In contrast to adult cardiomyocytes, proliferation of cardiomyocytes is robust during embryonic and fetal development [4, 5]; even immediately after birth, more than 30 % of mouse cardiomyocytes can still be labeled by incorporation of nucleotides used to define passage of cells through the S-phase, such as BrdU or EdU [5]. While the reasons why the proliferative capacity of cardiomyocytes is blunted immediately after birth still escape our understanding [6], the manipulation of cardiomyocyte gene expression by gene transfer represents an appealing possibility to both understand the molecular mechanisms regulating cell cycle progression in these cells and to foster their replication for regenerative purposes [6, 7].

Recent evidence indicates that the cardiomyocyte cell cycle is controlled by the microRNA (miRNA) network (reviewed in refs. 8, 9). In particular, a set of miRNAs is directly involved in both the positive and negative regulation of cardiomyocyte proliferation. The inhibitor miRNAs include various miR-15 [10] and let-7 family members [11]. Among the positive regulators are the miR-17/92 [12] and miR-302/367 [13] clusters, as well as a series of other miRNAs recently identified by whole genome, high-throughput screenings [7]. Taken together, these findings clearly indicate that the replicative potential of cardiomyocytes can be effectively modulated by overexpressing or inhibiting these miRNAs by either delivering synthetic miRNA mimics or inhibitors, or by overexpressing, in cardiomyocytes, miRNA genes, or RNA sponges as intracellular miRNA decoys.

A vector class that displays particularly favorable properties for cardiomyocyte gene transfer is the one based on the small, defective, and non-pathogenic human parvovirus Adeno-Associated Virus (AAV). These vectors are genetically simple and display specific tropism for different post-mitotic cells, including cardiomyocytes (extensively reviewed in ref. 14).

In this Chapter, we report protocols for culture and gene transfer of primary rodent neonatal cardiomyocytes using AAV vectors and the assessment of their capacity to undergo DNA replication.

2 Materials

2.1 Tissue

Neonatal (p0-p5), male and female, wild-type C57 BL/6 mice, and neonatal (p0-p5) wild-type Wistar rat hearts.

2.2 Supplies

1. Sterile surgical forceps and scissors for surgical biopsies or animal tissue.
2. Stirring bar 3 × 8 mm.

3. 0.22 μm Stericup Durapore filter (Millipore).
4. Cell strainer 40 μm Nylon.
5. 50 ml falcon tube.
6. Hemocytometer.
7. 10 mm Petri dishes.
8. Primaria multiwall plates or Petri dishes.
9. Serological pipettes (25, 10, and 10 ml).
10. 10 ml Strippette wide tip.
11. Sterile tips (1000 and 200 μl).
12. Trypan blue (0.4 %).

2.3 Equipment

1. Inverted microscope.
2. Water bath.
3. Centrifuge.
4. Laminar biosafety hood.
5. CO_2 incubator.
6. Autoclave.
7. Motorized pipet controller.
8. Pipettes (1000, 200, and 20 μl).

2.4 Media, Buffers, and Solutions

1. Calcium and bicarbonate-free Hanks' balanced salt solution with Hepes (CBFHH), prepared with the following components: NaCl 137 mM, KCl 5.36 mM, $MgSO_4.7H_2O$ 0.81 mM, Dextrose 5.55 mM, KH_2PO_4 0.44 mM, $Na_2HPO_4.7H_2O$ 0.34 mM, HEPES 20.06 mM. Dissolve powders in ultrapure water (prepared by purifying deionized water to obtain a sensitivity of 18 MΩ cm at 25 °C) and bring pH to 7.5. Filter with 0.22 μm Stericup Durapore filter (Millipore) and store at 4 °C for maximum 1 week.
2. Digestion solution is made by 150 ml of CBFHH, 0.2625 g of Trypsin (BD Difco) (final concentration 1.75 μg/ml), which is fundamental to disrupt extracellular matrix, and DNase I from bovine pancreas (Sigma) which is used during the isolation to prevent cell clumping by digesting sticky DNA released from lysed cells. A DNase stock solution is prepared by dissolving it in 0.15 M NaCl to obtain a concentration of 2 mg/ml. After filtration with 0.22 μm Stericup Durapore filter (Millipore), 1 ml aliquots can be stored at −20 °C. For 150 ml of CBFHH, use 1.5 ml of 2 mg/ml DNase solution (final concentration is 20 μg/ml). Trypsin and DNase should be added to CBFHH immediately before the experiment. The volume of digestion solution is prepared according to the number of hearts to be digested.

3. Fetal Bovine Serum (FBS).
4. Complete medium is made by Dulbecco's modified eagle medium (DMEM), 4.5 g/l Glucose, with 5 % FBS, 100 U/ml penicillin and 100 μg/ml streptomicin, 20 μg/ml vitamin B12 (Sigma). Vitamin B12 can be earlier resuspended in MilliQ water to obtain a solution stock of 2 mg/ml, filtered and stocked in 1 ml aliquots.
5. Phosphate buffered saline (PBS) without calcium and magnesium.

2.5 Transfection Reagents

1. miRNA mimics (Dharmacon).
2. siRNA buffer 5× (Dharmacon).
3. OPTIMEM (Life Technologies).
4. RNAiMax Lipofectamine (Life Technologies).

2.6 Antibodies and EdU Assay

1. Anti-alpha actinin antibody (Abcam).
2. Anti-BrdU (Abcam).
3. Anti-phospho Histone H3 (Ser 10) (Millipore).
4. Anti-aurora B kinase (Sigma).
5. Anti-mouse (fluorescent) (Life Technologies).
6. Anti-rat(fluorescent) (Life Technologies).
7. Hoechst (Life Technologies).
8. Click-it EdU Alexa Imaging kit (Life Technologies).

3 Methods

3.1 Isolation of Neonatal Rat and Mouse Cardiomyocytes (See Notes 1, 2, and 3)

All the following steps must be performed under laminar biosafety hood wearing lab coat and clean gloves.

1. Euthanize pups by beheading and open the chest using clean surgical forceps and scissors (*see* **Note 4**). To avoid cell contamination, clean the skin of pups with unidirectional movements from the neck to the abdomen using alcohol pads before sacrifice.
2. With sterile surgical forceps and scissors, explant hearts and transfer in sterile and cold (4 °C) CBFHH solution, squeeze with forceps force bleeding; remove with sterile instruments vessels and debris.
3. Cut each heart into small pieces and transfer them into a sterile 50 ml Falcon tube. Let the heart pieces settle in the bottom of the tube and aspirate excess of CBFHH.
4. Add a 3 × 8 mm stirring bar (rinsed in advance in ethanol and subsequently in PBS) to the Falcon tube.

5. Add 10 ml of sterile digestion solution at room temperature and pipet 10 times with 10 ml Strippette wide tip, setting the motorized pipet controller at medium speed to ensure adequate chunk separation.
6. Place a magnetic stirrer at 37 °C and place the Falcon tube with the heart pieces on top. Set it at medium speed for 10 min.
7. Under a laminar biosafety hood, pipet again 10 times. Discard as much volume as you can. Be careful not to discard the heart chunks. This first digestion is just a wash step to remove red cells and debris.
8. Repeat **steps 5** and **6**.
9. Aspirate as much supernatant as you can and transfer it into a new sterile 50 ml Falcon tube containing 7 ml of sterile fetal bovine serum (FBS) at room temperature. The supernatant contains mainly cardiomyocytes and fibroblasts.

   After each digestion, you should be able to recover a volume of 8–9 ml. Add subsequent digestions to the Falcon tube containing 10 ml of FBS to reach a final volume of 50 ml (*see* **Note 5**).
10. Repeat **steps 8** and **9** several times for a total timespan of 2–3 h or until almost all the tissue is digested (*see* **Note 6**). While performing the last digestions, volumes and time can be decreased to 7 ml and 5 min respectively.
11. Collected cells by centrifugation at 330RCF for 10 min at room temperature.
12. Aspirate supernatant and resuspend the pellet with 20–40 ml of complete medium at 37 °C. The volume amount depends on the number of starting pups. For example, a volume of 20 ml is appropriate for 7–8 p1 rat pups.
13. Pass the collected cells through a 40 μm cell strainer (this allows one to avoid to plate extracellular matrix debris and cellular aggregates) and plate the cells in two 10 mm non-primary Petri dishes (10 ml of resuspended cells each).
14. Place cells in the incubator (37 °C, 5 % CO_2, humidified atmosphere) for 2 h. During this step, the non-myocyte components (mainly fibroblasts) attach to the plate.
15. After **step 14**, the supernatant contains mainly cardiomyocytes that are now enriched over the non-myocyte cell population (around 95 % purity). They can now be counted using a hemocytometer and seeded at the desired concentration on primaria plastic (*see* **Notes 7** and **8**).
16. Rat and mouse cardiomyocytes are seeded according to the numbers in Table 1, which considers maintaining cells in culture for 96 h.
17. Check the cells 24 h after plating and change the medium every 48 h (*see* **Note 9**).

Table 1
Number of rat and mouse cardiomyocyte to be seeded in different multiwell plates

	N. cardiomyocytes		
	96 multiwell	*24 multiwell*	*6 multiwell*
Rat	15,000	100,000	500,000
Mouse	30,000	200,000	1,000,000

3.2 Transduction of Cardiomyocytes with AAV Vectors

1. Neonatal rodent cardiomyocytes can efficiently be transduced with viral vectors based on the adeno-associated virus serotype 6 (AAV6) (*see* **Note 10**) immediately after isolation or the day after prior to a medium change to remove the dead cell debris (*see* **Note11**).
2. Transduce cells by adding 1×10^4 viral particles per cell to the medium, following the formula:

$$\frac{(\text{number of seeded cells}) \times (1 \times 10^4 \text{ viral particles per cell})}{(\text{viral titer})}$$

   Example: 100.000 cells have been seeded and the viral titer is 1×10^{12} viral particles/ml.

$$\frac{(100.000) \times (1 \times 10^4)}{(1 \times 10^{12})} = 0.001\text{ml}$$

3. Incubate cells overnight, even if virus internalization is completed in 3–4 h.
4. Change medium with fresh one containing all supplements.

3.3 Transfection of Cardiomyocytes with Small RNAs (See Notes 12 and 13)

1. For maximum transfection efficiency, cardiomyocytes should be at 70 % confluence (with this protocol, 90–95 % of transfection efficiency is commonly achieved) (*see* **Note 14**).
2. Change medium and add fresh medium without penicillin and streptomycin.
3. Prepare a master mix (Table 2) with serum-free medium OPTIMEM and Lipofectamine RNAiMax, a cationic lipid especially suitable for small RNA transfection. Incubate 5 min at room temperature.

   Example: for a 96 multiwell plate, in order to achieve a final concentration of 50 nM, prepare for each well 34.6 µl OPTIMEM and 0.4 µl Lipofectamine RNAiMax.
4. Dilute the small RNA (e.g., siRNA, miRNA mimics, or inhibitor) in siRNA buffer according to Table 2.

Table 2
Transfection reagent volumes to obtain 25 nM or 50 nM small RNA final concentration for transfection of neonatal rat cardiomyocytes

	96 Multiwell	*24 Multiwell*	*6 Multiwell*
Final concentration:	**25 nM**		
small RNA (20 μM) (μl)	0.2	0.5	1.6
siRNA buffer (μl)	7.3	21.9	65.8
RNAiMax (μl)	0.2	0.6	1.8
OPTIMEM (μl)	42.3	126.9	380.7
Add to each well (μl)	50	150	450
Medium (μl)	100	300	900
Final concentration:	**50 nM**		
small RNA (20 μM) (μl)	0.4	1.1	3.3
siRNA buffer (μl)	14.6	43.9	131.7
RNAiMax (μl)	0.4	1.2	3.6
OPTIMEM (μl)	34.6	103.8	311.5
Add to each well (μl)	50	150	450
Medium (μl)	100	300	900

Example: for a 96 multiwell plate, to have a final concentration of 50 nM, prepare for each well 0.4 μl miRNA or siRNA 20 μM and 14,6 μl siRNA buffer.

5. Mix OPTIMEM and Lipofectamine RNAiMax with the small RNA and siRNA buffer (Table 2). Incubate for 20–30 min at room temperature.
6. Dispense the mixture onto the cells obtained in the previous step.
   For 96 multiwell plates, dispense 50 μl per well.
7. After 6 h, check cell viability and change the medium with fresh one containing all supplements

3.4 Evaluation of Cardiomyocyte Proliferation by Thymidine Analogue Incorporation

Cardiomyocyte proliferation can be evaluated by assessing incorporation of thymidine analogues such as 5-bromo-2′-deoxyuridine (BrdU) or 5-ethynyl-2′-deoxyuridine (EdU) during active DNA synthesis. BrdU detection is an antibody-based technique that requires DNA denaturation, while EdU assessment is based on a copper-catalyzed covalent reaction between an azide and an alkaline (Click chemistry) without the need of DNA denaturation.

3.4.1 Evaluation of Cardiomyocyte Proliferation by EdU Incorporation

1. When cells reach the desired density remove medium and replace it with complete medium with 10 μM EdU. Edu stock is 10 mM in DMSO and has to be stored at −20 °C.
2. Incubate cells for 20–24 h (*see* **Note 15**).
3. Remove medium and wash cells briefly with PBS.
4. Fix cells with 4 % formaldehyde for 15 min at room temperature.
5. Discard fixative and wash three times, 10 min each, at room temperature with PBS.
6. Permeabilize cells by incubating for 20 min at room temperature with 0.5 % Triton X-100 in PBS.
7. Remove permeabilizing solution and block with 1 % Bovine Serum Albumin in PBS for 1 h at room temperature.
8. Incubate with anti-sarcomeric α-actinin antibody 1:150 in 1 % Bovine Serum Albumin in PBS, overnight at 4 °C (*see* **Note 16**).
9. Aspirate primary antibody and wash three times, 10 min each, at room temperature with PBS.
10. Incubate with appropriate fluorescent secondary antibody (anti-mouse 1:500) in 1 % Bovine Serum Albumin in PBS, 2 h at room temperature protected from light.
11. Discard secondary antibody and wash three times, 10 min each protected from light, at room temperature with PBS.
12. Prepare 1× Click-iT EdU Reaction Buffer Additive and 1× Click-iT EdU Reaction Buffer by diluting the 10× solution 1:10 in deionized water. Prepare this solution fresh and use it on the same day.
13. Prepare Click-iT reaction cocktail according to Table 3. It is important to add the ingredients in the order listed in the Table; otherwise, the reaction will not proceed optimally. Use the Click-iT reaction cocktail within 15 min from preparation.

Table 3
Click-it reaction cocktail for the incorporation of Edu

Reaction components:	Total volume		
	500 μl	1 ml	2 ml
1× Clic-it Reaction buffer	430 μl	860 μl	1.8 ml
$CuSO_4$	20 μl	40 μl	80 μl
Alexa Fluor Azide	1.2 μl	2.5 μl	5 μl
Reaction Buffer Additive	50 μl	100 μl	200 μl

14. Incubate 30 min at room temperature protected from light.
15. Remove the Click-it reaction cocktail and wash three times protected from light, 10 min each, at room temperature with PBS.
16. Incubate in the dark with Hoechst 1:1,500 in PBS, at room temperature for 20 min to stain nuclei.
17. Now plates are ready to be acquired by high content microscopy and quantified. They can be stored for a few weeks at 4 °C protected from light (*see* **Note 17**).

3.4.2 Evaluation of Cardiomyocyte Proliferation by BrdU Incorporation

1. Remove medium and replace it with fresh medium with all supplements including BrdU at a final concentration of 10 μM.
2. Incubate cells for 20–24 h. This time is usually sufficient to detect all proliferating cells traveling through the S-phase.
3. Discard the medium and wash briefly with PBS.
4. Fix cells with 4 % formaldehyde for 15 min at room temperature.
5. Eliminate the fixative and permeabilize three times at room temperature with 1 % TritonX-100 in PBS.
6. Denature DNA by adding HCl 1 N and keep the plate for 10 min above ice. Immediately afterward, discard and replace with HCl 2 N; incubate for 20 min at 37 °C.
7. Neutralize with NaBorate 0.1 M pH 8.4 for 12 min at room temperature.
8. Discard NaBorate and wash three times at room temperature with 1 % Triton X-100 in PBS.
9. Block for 1 h at room temperature with 2 % BSA in PBS.
10. Incubate with primary antibodies against sarcomericα-actinin (1:150) and against BrdU (1:200) in blocking solution overnight at 4 °C.
11. Discard the primary antibodies and wash three times, 10 min each, at room temperature with 0.2 % Tween in PBS.
12. Incubate with the appropriate fluorescent secondary antibodies (anti-mouse 1:500 to detect sarcomeric α-actinin and anti-rat 1:1000 to detect BrdU) in blocking solution for 2 h at room temperature protected from light.
13. Remove secondary antibody and wash three times at room temperature with 0.2 % Tween in PBS protected from light.
14. Incubate the plate covered from light with Hoechst 1:1500 in PBS, at room temperature for 20 min to stain nuclei.
15. Now plates are ready to be quantitatively analyzed by high content microscopy (Fig. 1a and b). They can be stored at 4 °C for some weeks protected from light.

3.5 Evaluation of Cardiomyocyte Proliferation by Phospho-Histone H3 and Aurora-B Staning

Additional information to assess cardiomyocyte proliferation is by staining cells for the proliferation markers phospho-histone 3 (pH 3) and Aurora B kinase. H3 phosphorylation on serine 10 is a crucial event for mitosis and appears early in the G_2 phase within pericentrometric heterocromatin, to later spread in an ordered fashion coincident with mitotic chromosome condensation (Fig. 1c). Aurora B localization in midbodies is instead specific for the M phase, when it regulates chromosome segregation and the spindle checkpoint (Fig. 1d).

The antibodies against pH 3(Ser10) and Aurora B are both used at 1:100 dilution.

1. Remove medium and wash cells briefly with PBS.
2. Fix cells with 4 % formaldehyde for 15 min at room temperature.

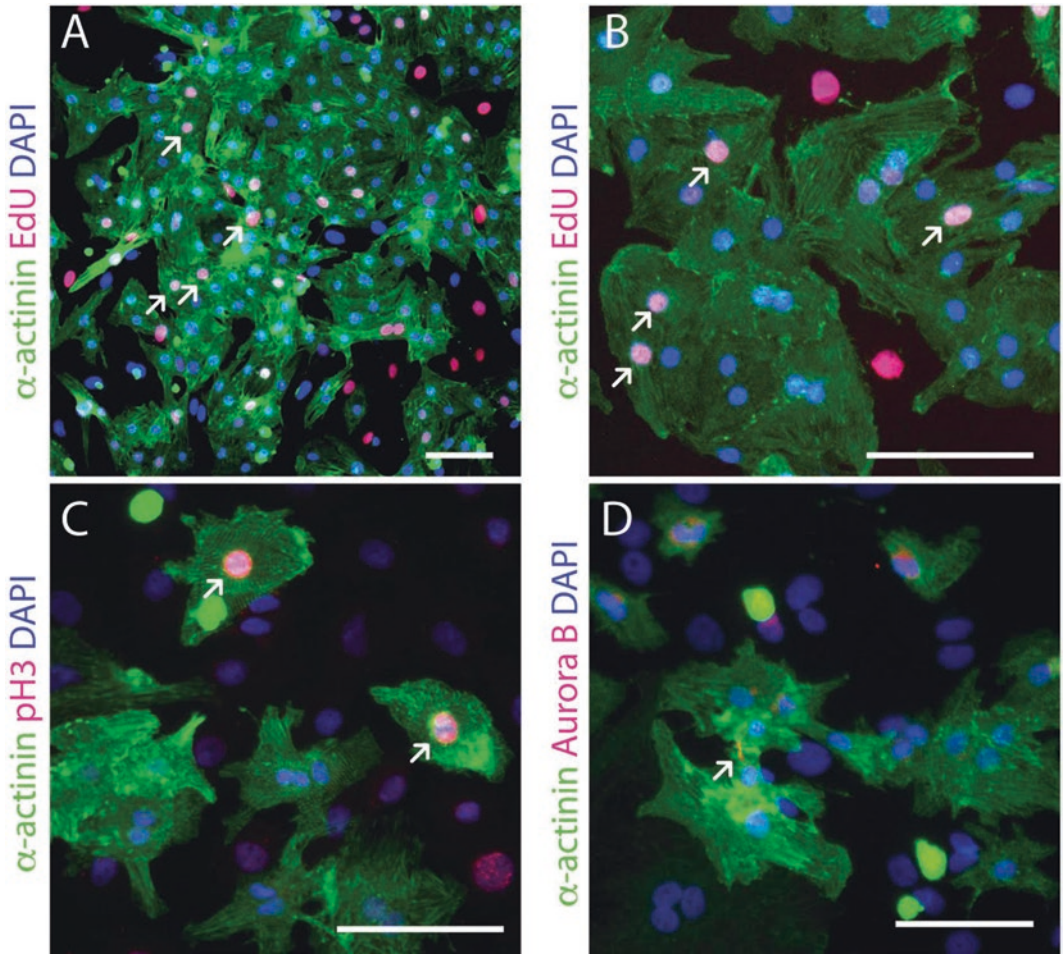

Fig. 1 Representative immunofluorescence pictures of rat neonatal cardiomyocytes. (**a**) and (**b**) Detection of DNA synthesis by EdU incorporation. Cells are stained in *green* to detect a-actinin, nuclei are in *blue*, proliferating nuclei in *red* (representative nuclei indicated by *arrows*). (**c**) Cells positive for histone 3 phoshorylated at serine 10 (*arrows*). (**d**) Aurora B localization in midbodies (*arrow*). Scale bars: 100 μM

3. Discard fixative and wash three times, 10 min each, at room temperature with PBS.
4. Permeabilize cells by incubating for 20 min at room temperature with 0.5 % Triton X-100 in PBS.
5. Remove permeabilizing solution and block with 1 % Bovine Serum Albumin in PBS for 1 h at room temperature.
6. Incubate with the primary antibodies against sarcomeric α-actinin (1:150), pH 3(Ser 10) (1:100), or Aurora B (1:100) in 1 % Bovine Serum Albumin in PBS, overnight at 4 °C.
7. Remove primary antibody and wash three times, 10 min each, at room temperature with PBS.
8. Incubate with appropriate fluorescent secondary antibody (anti mouse 1:500) in 1 % Bovine Serum Albumin in PBS, 2 h at room temperature protected from light.
9. Discard secondary antibody and wash three times, 10 min each protected from light, at room temperature with PBS.
10. Incubate the plate covered from light with Hoechst 1:1500 in PBS, at room temperature for 20 min to stain nuclei.
11. Now plates are ready to be quantitatively analyzed by high content microscopy. They can be stored at 4 °C for some weeks protected from light.

4 Notes

1. The procedure to isolate neonatal rat and neonatal mouse cardiomyocytes is almost identical. The number of hearts normally used is higher for mice than for rats, due to relative heart size and number of cardiomyocytes that can be recovered.
2. This protocol refers to a number of rat pups between 7 and 12; if a different number of animals are used, volumes and digestion steps should be adapted accordingly. In the case of mice, the number of pups is between 10 and 20.
3. For a successful isolation, pups from both species, regardless of sex, should be between day 0 (p0) and day 5 (p5).
4. All animal procedures should be conducted in accordance with animal care policies and must be approved by the appropriate institutional animal care committees.
5. FBS is fundamental to inactivate trypsin and allow a proper recovery of the cells. During digestion, FBS-containing cells should be placed at 37 °C.
6. At the end of each digestion step, the solution turns turbid. This is an indication of appropriate digestion.
7. Vital cells can be counted by adding, to the medium, a volume of 0.4 % Trypan Blue that is equal to the volume in which the

cells are contained. Viable cardiomyocytes are large, rounded, transparent and do not stain with the dye.

8. The expected yields of this procedure are approximately 2 and 1 million cells for each rat and mouse pup respectively.
9. Twenty-four hours after plating cells lose their rounded morphology, spread and start beating. A medium change 24 h after plating is recommended to remove debris and dead cells.
10. Adeno-associated-virus vector serotype 6 is the most recommended for efficient in vitro rodent cardiomyocyte transduction.
11. The presence of serum in the medium does not negatively affect transduction efficiency.
12. Cardiomyocytes can be transfected immediately after seeding (reverse transfection) or the day after, when cells are already attached (forward transfection). In both cases cells should be, at the moment of transfection, in antibiotic-free medium, since the presence of antibiotics negatively affects transfection efficiency.
13. miRNA transfection protocol of rat and mouse neonatal cardiomyocytes differs only for the final concentration: 25 nM for rat and 50 nM for mouse cardiomyocytes. As far as siRNAs are concerned, the proper concentration should be tested experimentally on a case-by-case basis.
14. Small Petri dishes (e.g., 35 mm dishes) or small-well multiwell (e.g., 96 multiwell) plates are usually preferred for transfection, in order to use lower amounts of RNA and transfection reagents.
15. EdU incubation timespan depends on each cell-type growth rate. A 20-h incubation time is commonly sufficient to detect cardiomyocyte proliferation.
16. Cardiomyocytes need to be specifically recognized from fibroblasts by immunofluorescence against a specific marker, such as troponin T, sarcomeric α-actinin, myosin heavy chain, or others.
17. EdU detection may cause high background or precipitates. If so, wash extensively several times with PBS or PBS 0.2 % Tween.

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Chapter 5

Assessment of Energy Metabolic Changes in Adipose Tissue-Derived Stem Cells

Ghazaleh Hajmousa and Martin C. Harmsen

Abstract

Adipose tissue-derived stem cells (ADSC) are promising candidates for therapeutic applications in cardiovascular regenerative medicine. By definition, the phenotype ADSCs, e.g., the ubiquitous secretion of growth factors, cytokines, and extracellular matrix components is not met in vivo, which renders ADSC a culture "artefact." The medium constituents therefore impact the efficacy of ADSC. Little attention has been paid to the energy source in medium, i.e., glucose, which feeds the cell's power plants: mitochondria. The role of mitochondria in stem cell biology goes beyond their function in ATP synthesis, because it includes cell signaling, reactive oxygen species (ROS) production, regulation of apoptosis, and aging. Appropriate application of ADSC for stem cells therapy of cardiovascular disease warrants knowledge of their mitochondrial phenotype and function. We discuss several methodologies for assessing ADSC mitochondrial function and structural changes under environmental cues, in particular, increased ROS caused by hyperglycemia.

Key words ADSC, Mitochondria, Hyperglycemia, ROS, Energy metabolism

1 Introduction

Adipose tissue-derived stem cells (ADSC) are a promising source of white adipose tissue stromal cells for use in cardiovascular regenerative medicine; for their differentiation potential, their ease of isolation and their secretion of therapeutically relevant trophic factors [1, 2]. The therapeutic potential of ASCS has been assessed in various animal models with specific disorders such as Parkinson's disease [3] and Alzheimer's disease [4, 5], bone and cartilage defects [6, 7], skin wound healing [8], myocardial infarction [9, 10], and diabetic retinopathy [11, 12].

There is new evidence that, in addition to growth factors and extracellular matrix cues, the (energy) metabolism of stem cell directs self-renewal and differentiation [13–15].

Recent studies have revealed limitations in the therapeutic efficacy of ADSC derived from patients who were compromised by

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diabetes or aging or obesity. It has been demonstrated that ADSC from these patients have impaired differentiation and migration [16–19]. Recently, we also showed in vitro that ADSC respond to chronic hyperglycemic exposure by increased apoptosis caused by amplified ROS. In addition, hyperglycemically cultured ADSC showed an altered mitochondrial membrane potential and changes in mitochondrial network morphology. Interestingly, we found an altered glycolysis and glucose uptake potential in ADSC upon culture under hyperglycemic conditions (30 mM D-glucose) compared with normaglycemically (5 mM D-glucose) cultured ADSC. These data confirm the well-established fact that mitochondrial disorders have a key role in apoptosis [20] and it contributes to a wide number of diseases, including mitochondrial myopathies [21], mitochondrial neuropathies [22], and diabetes [23]. Mitochondria are a main source of reactive oxygen species (ROS) in the cell [24]. In healthy cells, the inner membrane of mitochondria is impermeable to ions [25] which allows the electrons transport chain (ETC) to build up the proton gradient required to generate energy. The mitochondrial membrane potential ($\Delta\Psi_m$) results from the difference in electrical potential generated by the electrochemical gradient across the inner membrane [26]. Mitochondria are the source for ROS, but also the major target of their damaging effects, demonstrating the trigger for several mitochondrial dysfunctions. Chronic increases in ROS production cause the accumulation of ROS-associated damage in DNA, proteins, and lipids, and are headed by severe perturbations in mitochondrial function detected as a decrease in $\Delta\Psi_m$. This reduction in $\Delta\Psi_m$ is accompanied by the production of ROS contributing to cell apoptosis [27].

Alterations of the glucose metabolism may cause mitochondrial dysfunction, i.e., affect the energy metabolism, and may be responsible for further cellular damage and disease pathogenesis. The failure to manage cellular energy pathways either the aerobic respiration or glycolysis via mitochondria may result in serious complications in diseases such as diabetes [28]**.** Detecting mitochondrial dysfunction in therapeutic used ADSC is a prerequisite in the development of novel stem cell therapies for diseases such as diabetes.

2 Materials

2.1 Isolation of ADSC

1. Human subcutaneous fat tissue or liposuction-derived fat.
2. Phosphate-buffered saline (PBS).
3. PBS/1 % Bovine serum albumin (BSA).
4. 0.1 % Collagenase dissolved in PBS/1%BSA, freshly prepared prior to use (Dissociation medium).

5. Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 100 U/ml Penicillin, 100 μg/ml Streptomycin, 2 mM L-glutamine, and 1 (gr/L) D-glucose (control medium). Store at 4 °C.
6. 50-ml Centrifuge tube.
7. 40-μm Nylon mesh.
8. Lymphoprep.
9. Lysisbuffer.
10. Trypan blue.

2.2 ADSC Culture

1. DMEM supplemented with 10 % fetal bovine serum (FBS) and 100 U/ml Penicillin, 100 μg/ml Streptomycin, 2 mM l-glutamine, and 1 (gr/L) D-glucose (control medium) or 4.5 (gr/L) D-glucose (hyperglycemic medium). Store at 4 °C.
2. Trypsin (0.25 %) and ethylenediaminetetraacetic acid (EDTA, 1 mM).

2.3 Apoptosis Detection of ADSC (Using FACS Calibur Flow-Cytometer)

1. Annexin V-Ethidium Homodimer lll (EthD-III): Apoptotic/Necrotic Cells Detection Kit (#PK-CA707-30018-Promokine).
2. Trypsin (0.25 %) and ethylenediaminetetraacetic acid (EDTA, 1 mM).
3. FACS tubes.

2.4 Intracellular ROS and Mitochondrial ROS Measurement in ADSC (Using FACS Calibur Flow-Cytometer)

1. 2′,7′-Dichlorofluorescin diacetate: H_2DCFDA (Thermo Fisher).
2. MitoSOX™ Red: Mitochondrial superoxide indicator (Thermo Fisher).
3. Trypsin (0.25 %) and ethylenediaminetetraacetic acid (EDTA, 1 mM).
4. Control medium/PBS.

2.5 Monitoring Mitochondrial Health

2.5.1 Assessment of Mitochondrial Membrane Potential

1. MitoProb ™ JC1 (5′,6,6′-tetrachloro-1,1′,3,3′tetraethylbenzimidazolylcarbocyanine iodide(Thermo Fisher)).
2. CCCP (carbonyl cyanide 3-chlorophenylhydrazone).
3. Trypsin (0.25 %) and ethylenediaminetetraacetic acid (EDTA, 1 mM).
4. Control medium/PBS.

2.5.2 Mitochondrial Morphology Analysis

1. Mito-Tracker Green [MTG] (Thermo Fisher).
2. Trypsin (0.25 %) and ethylenediaminetetraacetic acid (EDTA, 1 mM).
3. Control medium/PBS.

2.6 ADSC Bioenergetics Profiling

1. V7-PS XF24 cell culture microplates (Seahorse Bioscience), XF24 extracellular flux assay kits (Seahorse Bioscience).
2. DMEM-XF containing, 1 mM glutamine, 1 % FBS, 1 (gr/L) D-glucose (control medium), or 4.5 (gr/L) D-glucose (hyperglycemic medium) and pyruvate-free (Unbuffered medium).
3. Oligomycin (Seahorse Bioscience).
4. FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone).
5. Rotenone and Antimycin A.
6. 2-deoxy-D-glucose.
7. Pierce™ BCA Protein Assay Kit (Thermo Fisher).

2.7 Assessment of Glucose Uptake

1. 2-Deoxy D-glucose.
2. PBS.
3. DMEM supplemented with 10 % fetal bovine serum (FBS) and 100 U/ml Penicillin, 100 μg/ml Streptomycin, 2 mM l-glutamine, and 1(gr/L) D-glucose (control medium) or 4.5 (gr/L) D-glucose (hyperglycemic medium). Store at 4 °C (serum-free medium).
4. Insulin.
5. 2-Deoxy-D-[^{14}C]glucose (^{14}C-2-DOG).
6. NaOH.
7. β-Scintillation cocktail and β-scintillation counter.
8. Pierce™ BCA Protein Assay Kit (Thermo Fisher).

3 Methods

3.1 Isolation of ADSC

1. Mince the fat tissue with fine scissors in culture dishes, and transfer the material into a 50 ml centrifuge tube. Alternatively, lipoaspirated fat can be transferred to centrifuge tubes directly.
2. Wash the fat three times with PBS, centrifuge at 300 × *g* for 3 min each time.
3. Add an equal volume of dissociation medium with the fat, stir for 90 min in 37 °C water bath.
4. Filter the digested fat through 40-μm Nylon mesh; collect the flow-through in 50 ml tubes.
5. Centrifuge the cell suspension at 600 × g for 10 min to obtain a high-density ADSC pellet.
6. Aspirate the supernatant, being careful not to disturb the cell pellets.
7. Resuspend the cell pellets in 30 ml PBS/1%BSA and add the cell-suspension gently on the top of 15 ml Lymphoprep.

8. Centrifuge at 4 °C, 1000 × *g* for 20 min.
9. Carefully aspirate the cells from the interphase.
10. Resuspend cells in lysis buffer and place on ice for 5 min, centrifuge at 600 × *g* for 10 min.
11. Count the cells using Trypan blue and seed at a concentration of 1.25×10^5 cells/cm^2 in culture flasks.

3.2 ADSC Culture

1. Maintain the primary ADSC in control medium at 37 °C in 5 % carbon dioxide. Change the culture medium every 3 days.
2. Once adherent cells become confluent, aspirate the culture medium and wash the cells with 5 ml of PBS. Add 1–3 ml of trypsin–EDTA at 37 °C for 5 min to detach the cells.
3. Resuspend the ADSC with an equal volume of control medium.
4. Centrifuge the cell suspension at 300 × *g* for 10 min at 4 °C and split the cells 1:3 in control medium.
5. Use the cells from passage 2–7 for the experiments (e.g., to expose the cells to an apoptotic condition such as hyperglycemia).

3.3 Apoptosis Detection of ADSC (Using FACS Calibur Flow Cytometer)

1. Harvest ADSC from control/treated group: aspirate the culture medium and wash the cells with 5 ml of PBS. Add 1–3 ml of trypsin–EDTA at 37 °C for 5 min to detach the cells.
2. Wash the cells, centrifuge at 300 × *g* for 10 min at 4 °C, and resuspend the cells in 500 µl binding buffer (100,000 cells/500 µl) in FACS tubes.
3. Stain the cells with 2.5 µl FITC-Annexin V (marker for apoptosis) and 2.5 µl of Ethidium Homodimer lll (marker for necrosis) in the dark at room temperature for 15 min.
4. Analyze the samples (FITC-AnnexinV-Ex/Em= ~492/514 nm and EthD-III-Ex/Em= ~528/617 nm) using a FACS Calibur flow cytometer within 1 h after staining.

3.4 Intracellular ROS and Mitochondrial ROS Measurement in ADSC (Using FACS Calibur Flow-Cytometer)

1. Harvest ADSC from control/treated groups: aspirate the culture medium and wash the cells with 5 ml of PBS. Add 1–3 ml of trypsin–EDTA at 37 °C for 5 min to detach the cells.
2. Wash the cells, centrifuge at 300 × *g* for 10 min at 4 °C, and resuspend the cells in FACS tubes with 1 ml of warm control medium followed by incubation with 20 µM H_2DCFDA (*see* **Note 1**) or 5 µM MitoSOX™ Red (*see* **Note 2**) in the dark at 37 °C for 15 min.
3. Analyze the samples (DCF: Ex/Em= ~492/527 nm and oxidized MitoSOX: Ex/Em= ~510/580 nm) directly without washing, using a FACS Calibur flow-cytometer within 30 min after the staining.

3.5 Monitoring Mitochondrial Health

3.5.1 Assessment of Mitochondrial Membrane Potential

1. Harvest ADSC from control/treated groups: aspirate the culture medium and wash the cells with 5 ml of PBS. Add 1–3 ml of trypsin–EDTA at 37 °C for 5 min to detach the cells.
2. Wash the cells, centrifuge at 300 × *g* for 10 min at 4 °C and resuspend the cells in 1 mL warm control medium at 1×10^6 cells/ml, followed by adding 2 μM MitoProbe JC-1 and 50 μM CCCP as control.
3. Incubate the samples at 37 °C for 15 min.
4. Determine the mitochondrial accumulation of the probe by fluorescence emission shift from green (~529 nm) to red (~590 nm) by FACS Calibur flow-cytometer within 15 min after staining.

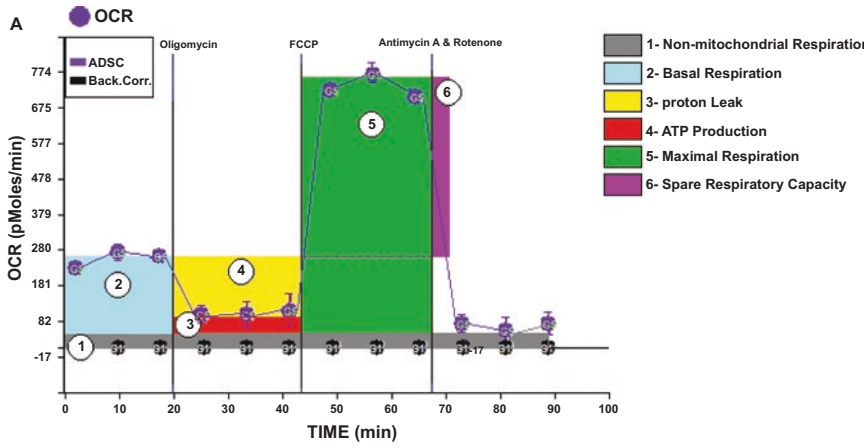
3.5.2 Mitochondrial Morphology Analysis

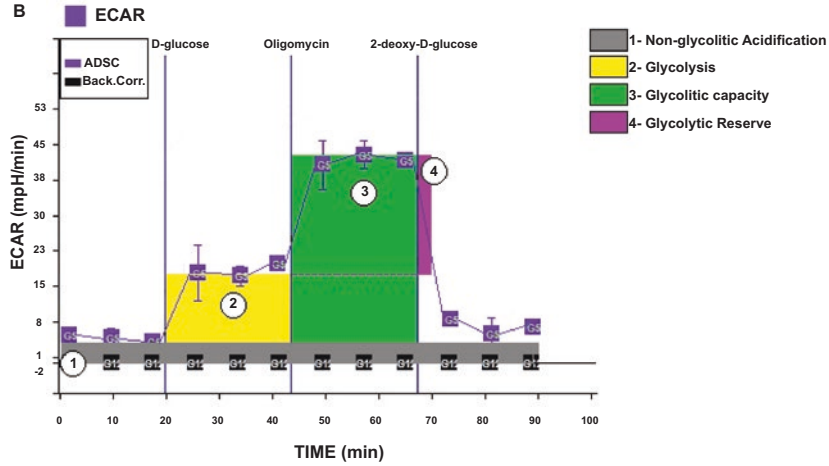
1. Once ADSC cultures from control/treated group become confluent, aspirate the culture medium and wash the cells with PBS twice.
2. Incubate the ADSC at 37 °C in a 5 % CO_2 humidified chamber with 120 nM membrane potential–independent dye: Mito-Tracker Green [MTG] in culture medium for 45 min.
3. Wash the cells three times with PBS and refresh the medium.
4. Keep the cells at 37 °C in a 5 % CO_2 humidified microscope stage chamber and image the cells live by confocal microscope with a 60× oil immersion objective (MTG: Ex/Em= ~488/550 nm).
5. Acquire in series of six slices per cell ranging in thickness from 0.5 to 0.8 μm per slice to visualize individual mitochondria as well as their interconnective network or disturbances.
6. Analyze the mitochondrial length and circularity (*see* **Note 3**) of ADSC with ImageJ software.

3.6 ADSC Bioenergetics Profiling

1. Plate the ADSC on V7-PS microplate under control or treatment condition to reach a confluent monolayer.
2. Wash the cells three times with PBS.
3. Replace the unbuffered medium and incubate cell at 37 °C in a CO_2-free incubator for 60 min.
4. For oxygen consumption rate (OCR (*see* **Note 4**)): pipet 2 μM oligomycin in port A, 5 μM FCCP in port B and a mixture containing 2 μM rotenone and 2 μM antimycin A in port C.

   For extracellular acidification rate (ECAR (*see* **Note 5**)): pipet the saturating concentration of glucose (10 mM) in port A, 2 μM oligomycin in port B, and 100 μM 2-deoxy-D-glucose in port C.
5. Place the microplate in XF24 Extracellular Flux Analyzer; Seahorse Bioscience to measure extracellular flux changes (Follow the protocol in Fig. 1c).
6. Normalize the results from the measurement to total cellular proteins in each well.





C

ADSC XF-analyzing protocol commands

EVENT	TIME	REPEAT
Start		
Loading Cartridge		
Calibration		
Equilibration		
Mixing	2 min	
Waiting	2 min	x3
Measuring	4 min	
Injecting From Port A		
Mixing	2 min	
Waiting	2 min	x3
Measuring	4 min	
Injecting From Port B		
Mixing	2 min	
Waiting	2 min	x3
Measuring	4 min	
Injecting From Port C		
Mixing	2 min	
Waiting	2 min	x3
Measuring	4 min	
End		

Fig. 1 Mitochondrial respiration graph of ADSC: Basal respiration, ATP production and proton leak after injecting oligomycin, maximal respiration after exposure the ADSC to FCCP and spare respiratory capacity of the cells were measured by using a Seahorse XF-analyzer and plotted (**a**). The glycolytic function of ADSC: glycolysis, glycolytic capacity after injecting oligomycin and glycolytic reserve are shown (**b**). Protocol commands for ADSC extracellular flux analyzing by Seahorse XF-analyzer (**c**)

3.7 Assessment of Glucose Uptake

1. Once ADSC cultures from control/treated group reach confluency, aspirate the culture medium and wash the cells twice with PBS.
2. Incubate the cells in serum-free medium at 37 °C for 4 h.
3. Stimulate the ADSC with 100 nM insulin for 20 min at 37 °C or leave untreated.
4. Remove the medium; wash the cells twice with warm PBS.
5. Add 1 ml of PBS containing 0.1 μCi 2-deoxy-D-[^{14}C]glucose (^{14}C-2-DOG) and unlabeled 100 μM 2-deoxy-D-glucose to each well.
6. Incubate the cells at 37 °C for 45 min.
7. Terminate the glucose transport by washing twice with ice-cold PBS.
8. Lyse the cells in 500 μl 0.05 M NaOH.
9. Use 400 μl of the aliquot for β-scintillation determination.
10. Use the remained 100 μl for the determination of protein concentration with the Pierce™ BCA Protein Assay Kit.

4 Notes

1. DCF formation is reflected to H_2O_2 production but it cannot be used to measure H_2O_2 production exclusively inside mitochondria. For imaging mitochondrial H_2O_2 in living cells we recommend peroxy-yellow-1 (MitoPY1), a new type of fluorophore [31], although advanced studies have to be performed to stablish its efficacy.
2. MitoSOX is considered a superoxide-specific probe to visualize superoxide ions inside mitochondria [29]. The MitoSox specify for hydrogen peroxide or reactive nitrogen species is quite low [30].
3. Mitochondrial circularity is a measure of the "roundness" of mitochondria with 0 referring to a straight line and 1 as a perfect circle. Cells containing a majority of long interconnected mitochondrial networks were classified as cells with tubular mitochondria. Cells with a majority of short mitochondria were classified as fragmented and cells with mostly sparse small round mitochondria were classified as very fragmented [32].

   As an example we show a mainly long and tubular mitochondrial network morphology in the healthy ADSC cultured in 5 mM D-glucose medium, which changed to a very fragmented morphology when cultured in medium with a non-physiologically high (50 mM) concentration of D-glucose (Fig. 2). The tubular mitochondria networks are desired for a normal function of the mitochondria by regulation of fusion

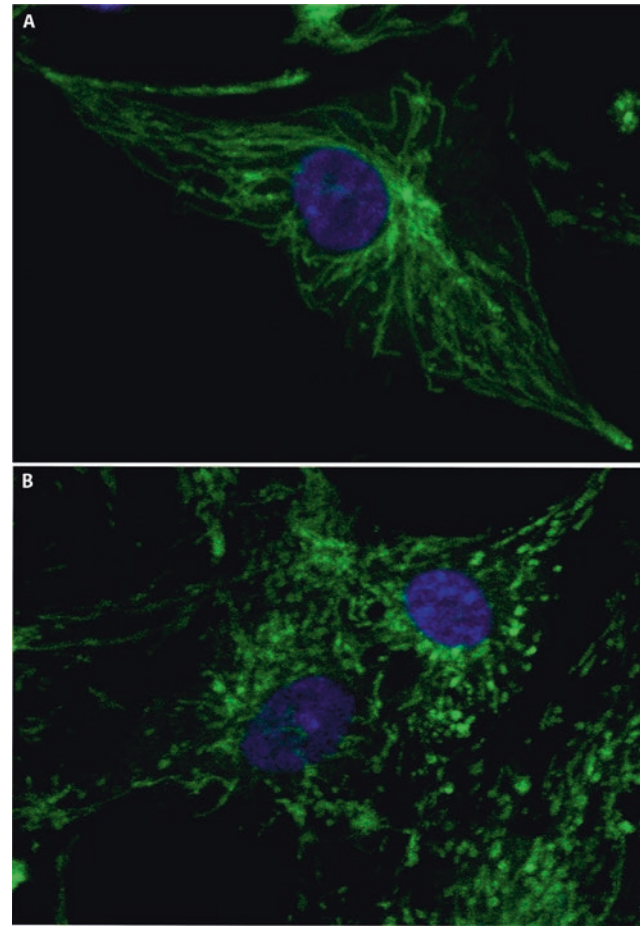


Fig. 2 Confocal immunofluorescent analysis of ADSC using staining with MitoTracker Green FM. (Blue pseudocolor = DAPI, nucleus staining). Long and tubular mitochondrial network morphology in healthy ADSC cultured in 5 mM D-glucose medium (**a**). Very fragmented mitochondrial morphology of ADSC after culturing in non-physiologically high (50 mM) D-glucose concentration medium (**b**)

and fission events that involve the formation or breaking of the mitochondria network, respectively. A decrease in the rate of fusion and a simultaneous increase in the rate of fission cause fragmentation of the mitochondrial network which results in shorter and rounder mitochondria [33, 34].

4. OCR measurement steps:
   (a) Measuring the basal OCR.
   (b) Inhibitory analysis using injections of oligomycin (Olig) at 2 μM which inhibits ATP synthase [ATP-linked respiration = $OCR_{pre\text{-}Olig} - OCR_{post\text{-}Olig}$], [proton leak = $OCR_{post\text{-}Olig} - OCR_{post\text{-}AntA/R}$].

(c) Applying proton ionophore FCCP at 5 μM, which uncouples mitochondria to obtain the maximum oxygen consumption rates [maximal respiration = $OCR_{post\text{-}FCCP} - OCR_{post\text{-}AntA/R}$], [respiratory capacity = $OCR_{post\text{-}FCCP} - OCR_{pre\text{-}Olig}$].

(d) Adding a mixture of an electron transport blocker, antimycin A (AntA) at 2 μM and rotenone at 2 μM as an inhibitor of mitochondrial complex to confirm that respiration changes were due mainly to mitochondrial respiration.

5. ECAR measurement steps:

(a) Measuring the basal ECAR in a medium without glucose or pyruvate.

(b) Measuring glycolysis rate of cells in saturating concentration of glucose [basic glycolysis = $ECAR_{pre\text{-}Olig}$].

(c) Inhibitory analysis using injections of oligomycin (Olig) at 2 μM which inhibits ATP synthase and shifts the energy production pathway to glycolysis to reach to the cellular maximum glycolytic capacity[glycolytic capacity = $ECAR_{post\text{-}Olig}$], [glycolytic reserve =$ECAR_{post\text{-}Olig} - ECAR_{pre\text{-}Olig}$].

(d) Inhibiting the glycolysis by using 100 μM2-deoxy-glucose, a glucose analog.

After normalization and analyzing the data, the mentioned mitochondrial respiration and glycolytic indexes can be calculated (Fig. 1a, b).

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Chapter 6

Isolation and In Vitro Characterization of Epidermal Stem Cells

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Abstract

Colony-forming assays represent prospective methods, where cells isolated from enzymatically dissociated tissues or from tissue cultures are assessed for their proliferative capacity in vitro. Complex tissues such as the epithelial component of the skin (the epidermis) are characterized by a substantial cellular heterogeneity. Analysis of bulk populations of cells by colony-forming assays can consequently be convoluted by a number of factors that are not controlled for in population wide studies. It is therefore advantageous to refine in vitro growth assays by sub-fractionation of cells using flow cytometry. Using markers that define the spatial origin of epidermal cells, it is possible to interrogate the specific characteristics of subpopulations of cells based on their in vivo credentials. Here, we describe how to isolate, culture, and characterize keratinocytes from murine back and tail skin sorted by surface antigens associated with adult stem cell characteristics.

Key words Keratinocyte, Isolation, Flow cytometry, Skin, Pilosebaceous unit, Stem cell

1 Introduction

1.1 Background, Rationale, and Relevance of the CFU Assay

How different stem cell subsets within complex tissues contribute to the maintenance of the adult organism remains an important question [1–5]. Closely related stem cell populations can be distinguished from each other based on parameters such as variability in marker expression, cell division rate, or resistance to disturbances within the local microenvironment (reviewed by [5]). Methods for clonal analysis are essential for understanding how stem cells arise during development, and how unique populations of cells contribute to maintenance during steady state homeostasis and regeneration. In vivo lineage tracing or fate mapping has emerged as the preferred assay for analyzing cell behavior in vivo [1]. In vitro assays are however important tools for quantifying and investigating many aspects of primitive cell behavior. Firstly, in vitro assays are required for analysis of cells of human origin [6]. Secondly, in vivo

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assays are relatively costly, time consuming and may be prone to technical- or biological limitations of the cells that are studied. Colony-forming assays were initially developed in the 1950s for the study of cell lines [7], a few decades later, it became an instrumental tool in stem cell biology [8].

The epidermis is the outer lining of the skin, which protects our organism against the hostile environment and from dehydration by forming an impermeable barrier. The epidermis can be divided into distinct compartments. The interfollicular epidermis (IFE) forms the protective barrier and attached to this outer lining of the skin are adnexal structures such as pilosebaceous units (PSU) that are composed of hair follicles and sebaceous glands, as well as apocrine and eccrine sweat glands (Fig. 1). The IFE is maintained by stem cells and progenitors that adhere tightly to the basement membrane. Constant proliferation within the IFE ensures the continuous replenishment of cells that are shed from the skin surface as dead squames. As cells lose their attachment to the basement membrane, they start expressing markers of terminal differentiation and will be lost from the system within a few weeks. Similar processes albeit at different speeds ensure the incessant turnover of cells in the sebaceous gland and in the upper permanent part of the PSU, known as the infundibulum and isthmus. In the lower part of the permanent PSU, stem cells and progenitor in the bulge are activated in a cyclic manner to regenerate hair follicles throughout life. Characterization of the PSU has identified extensive spatial confinement of numerous proteins with clearly defined boundaries [5, 9]. Fate-mapping studies have subsequently documented that the observed heterogeneity in cellular identity forms the basis for the maintenance of the PSU,

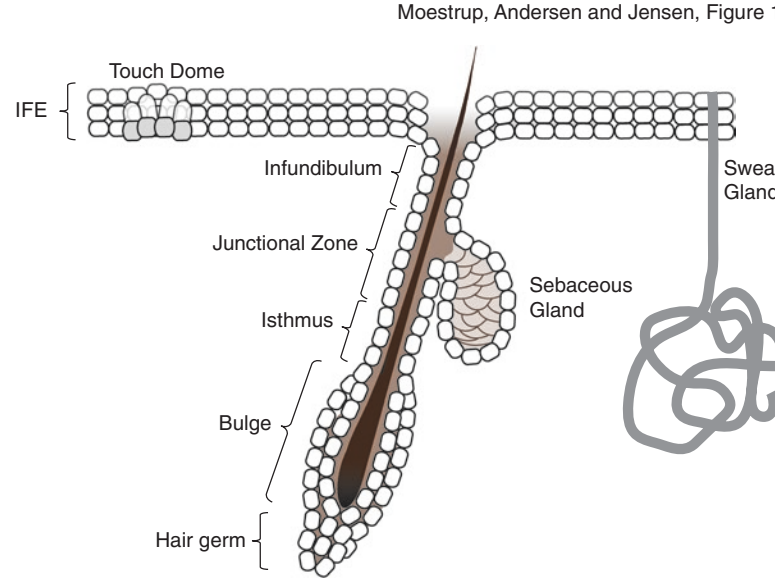


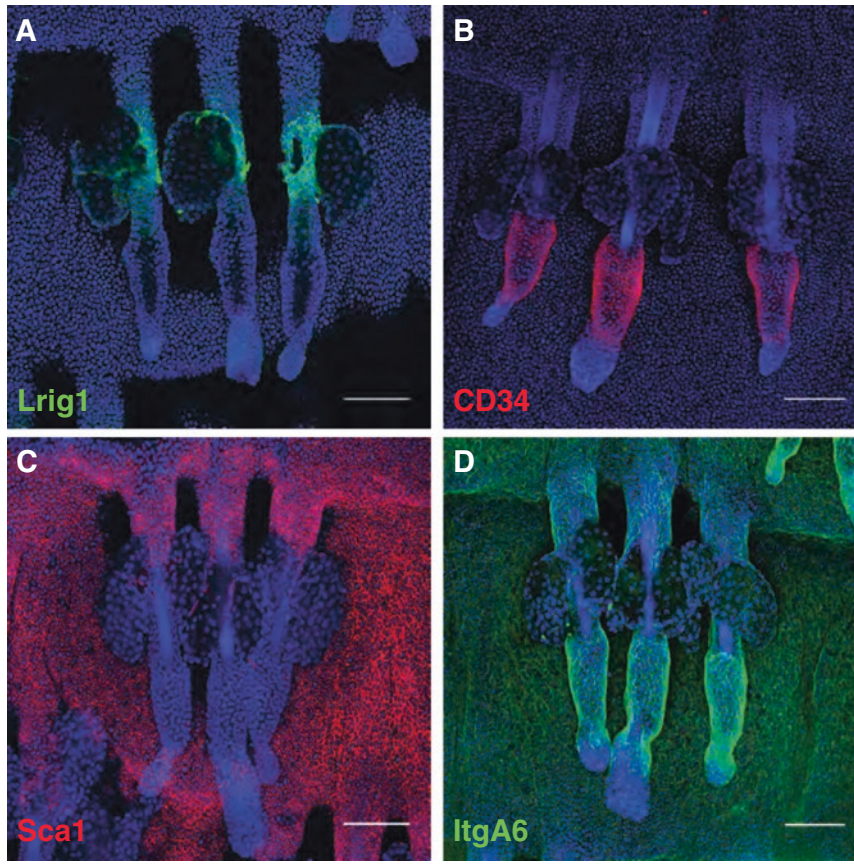
Fig. 1 Schematic illustration of the main components of the epidermis

as discrete populations of stem cells maintain different compartments with specialized functions in an autonomous manner [10]. Methods to assess the specific in vitro properties of these subsets of stem cells are however still in its infancy.

Clonal analysis of primary epidermal keratinocytes was pioneered by Barrandon and Green, using methods developed a decade earlier [11, 12]. This led to the first observation of cellular heterogeneity within the proliferative compartment of human skin and the identification of three different cellular subtypes. When seeded at clonal densities some cells gave rise to holoclones, which were large and rapidly growing colonies with a smooth parameter. Other cells gave rise to smaller colonies with a wrinkled perimeter, paraclones, or very small clones that were unable to be serially propagated, meroclones [12]. Importantly, holoclones were composed of cells that to a large fraction would form holoclones upon serial plating, whereas cells in paraclones would form a mixture of holoclones, paraclones, and meroclones. These results illustrate that cellular heterogeneity exists within populations of epidermal keratinocytes, that their differing growth potential can be assessed in vitro, and that the properties are inherited by daughter cells providing evidence for in vitro self-renewal of epidermal stem cells in the holoclones. A major reason for the continued interest in exploiting the colony-forming assay has come with the emergence of flow cytometry, which has advanced the procedures for isolating specific subpopulations of cells based on the expression of specific antigens ([13–15]; Table 1 and Fig. 2a–d).

Table 1
Compartment-specific markers associated with murine epidermis. The distinct localization is most profound during telogen phase of the pilosebaceous unit

Compartment	Marker profile	References
IFE & Infundibulum	Sca1+ Integrin α6+ CD34−	[16]
Junctional Zone	Lrig1+ Plet1+ Integrin α6low Sca1−	[17,18]
Isthmus	Gli1+ Lgr6+ Integrin α6low CD34− Sca1−	[20,21]
Bulge	CD34+ K19+ Lgr5+ (in bottom part only) Integrin α6high	[22–23]



E

	Average number of cells isolated	Hair follicle cycling	PSU Morphology
Tail skin	5x10^6	Always asynchronous	Large structures
Back skin	20x10^6	Syncronized	Small structures

F

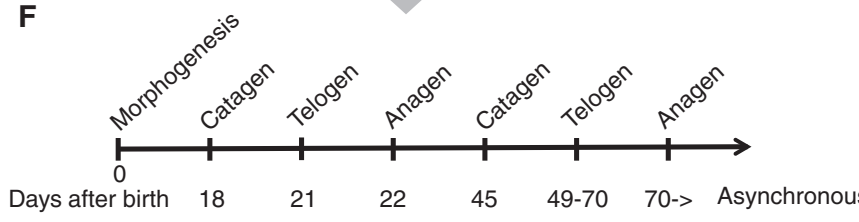


Fig. 2 Tail skin whole mounts stained for markers defining subsets of cells in murine epidermis. These markers have been used for multicolor flow cytometry to fractionate epidermal subpopulations localized into spatially confined compartments in telogen skin (Lrig1) (**A**—JZ), CD34 (**B**—Bulge), Sca1 (**C**—IFE and infundibulum) and Integrin Alpha6 (**D** (ItgA6)—epidermal keratinocytes). The image in **a** is kindly provided by Mahalia Page. (**E**) There are significant differences between mouse tail- and back skin, which need to be considered during experimental designs. This includes the total number of epidermal keratinocytes that can be isolated from an 8-week-old mouse, asynchronous versus synchronous hair growth, and the size of the PSU. (**F**) The timeline shows the different stages of hair follicle growth in mouse back skin. Once morphogenesis finishes around postnatal day 18, hair follicles will go through phases of regression (catagen), rest (telogen) and growth (anagen) for the rest of the animals' life. The phases are synchronous throughout the back skin until around postnatal week 10. Scale bar in **A**–**D** 100 μm

The readout for colony-forming assays should be carefully considered when planning how to isolate and process the cells following seeding. The different end-points of cultured epithelial cells include epithelial regeneration [15], long-term proliferative potential in vitro [11], or simply measuring colony-forming efficiency, colony size and morphology. In this chapter, we will focus on the three latter read-outs for epidermal keratinocytes isolated from mouse skin.

1.2 Tissue of Interest for Stem Cell Characterization and Isolation

An important consideration is that skin has unique properties depending on body site. Mouse back skin is the focus of most studies, as it provides an attractive source of tissue, where hair growth is synchronous for roughly the first 10 weeks after birth (Fig. 2e, f). Specific time points can be chosen accordingly to study the tissue in either growth or resting phase of the hair follicle. Although much less tissue, palmar plantar paw epidermis from mouse is a rich source for studies of IFE, since it is devoid of any PSUs. Moreover, hind paws are the only sites of sweat glands in the mouse. The mouse tail is also an attractive specimen for studies of the epidermis. Due to its thickness it is possible to generate whole mounts, where spatial arrangements within the epithelium can be studied at cellular resolution following dissociation of the epidermis from the underlying mesenchyme [24] (Fig. 2). However, the IFE of the tail has elements in common with reptiles as it following birth is patterned into a scale-like structure with distinct parakeratotic (scale) and orthokeratotic (interscale) regions. This makes tail epidermis unique [25]. Moreover, hair follicles are unlike those in back skin constantly cycling in an asynchronous manner, which makes it very difficult to perform controlled population-based studies on cells isolated from the tissue. Nonetheless, tail- and back skin remains the most widely studied skin types in mice for assessing stem cell heterogeneity within the epidermis. It is possible to isolate sub-populations of cells from epidermal cell suspensions with high purity based on cell surface markers or specific fluorescent mouse reporter strains (Fig. 3). Below, we describe the methodology to identify and isolate different fractions of stem cells that populate the PSU in mouse epidermis.

2 Materials

2.1 3T3 NIH Fibroblast-Coated Wells Components

1. 3T3 NIH fibroblast medium: DMEM GlutaMAX™, 10 % adult bovine serum Penicillin/streptomycin solution (100 U/mL penicillin-G and 100 μg/mL streptomycin).
2. 100× Mitomycin Caliquots:
   Dissolve 2 mg mitomycin C in 5 mL sterile H_2O. Aliquot and store at −20 °C.
3. T75 tissue culture flask.

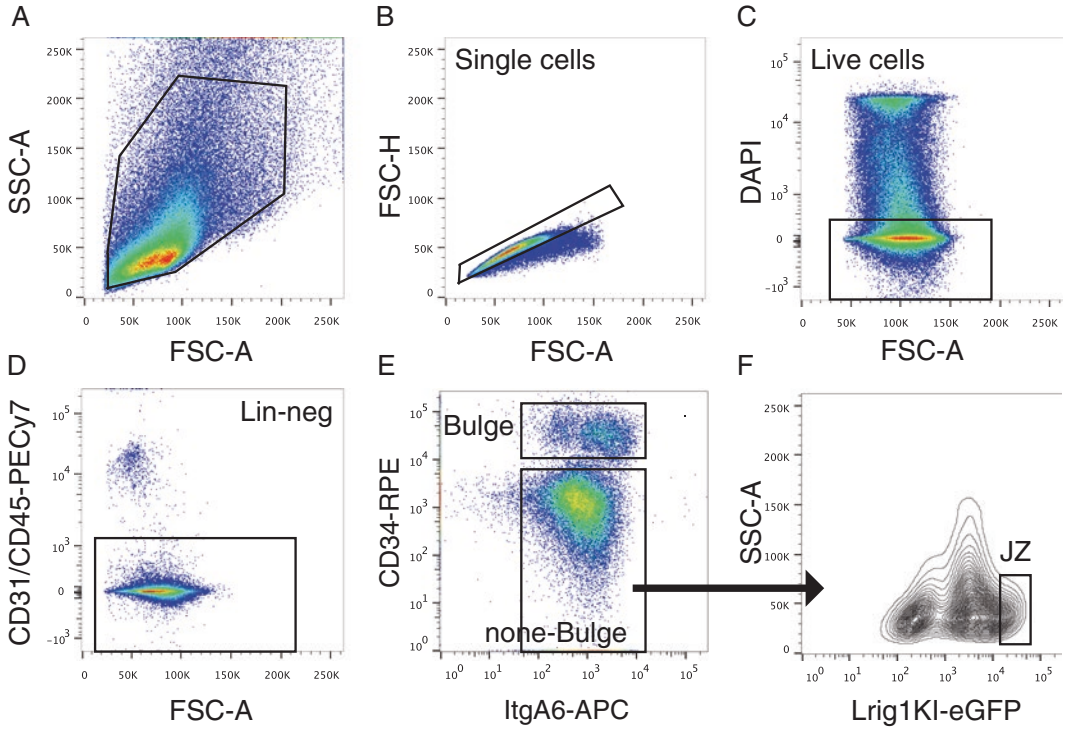


Fig. 3 Strategy for isolation of subpopulation of epidermal cells from back skin samples. (**A**) Cells are initially separated from debris using forward and sideward scatter (FSC and SSC, respectively). (**B**) Singlets are subsequently separated from doublets based on the relationship between forward scatter area and height. (**C**) In order to isolate live cells are separated based on dye exclusion. (**D**) Hematopoietic and endothelial cells are excluded based on CD45 and CD31 expression. (**E**) CD34 and ItgA6 can subsequently be used to separate hair follicle stem cells from the remaining epidermal keratinocytes based on expression of CD34. (**F**) Within the none-bulge population Lrig1 expressing cells can be identified based on either Lrig1 protein expression or GFP using a reporter mouse model

4. 6-well or 24-well plates.
5. Collagen Type 1, rat tail.
6. Phosphate buffered saline (PBS).
7. Versene solution (Thermofisher Scientific).
8. Trypsin (0.25 %, Thermofisher).

2.1.1 Tissue Disinfection and Isolation of Keratinocytes Components

1. Iodine: Dilute 1/300 in PBS and use immediately. It cannot be stored.
2. Ethanol: 70 % ethanol.
3. 10 % ABS: Dilute adult bovine serum 1:10 in sterile PBS and keep at 4 °C.
4. 70 μm cell strainer.

5. 50 mL Falcon tubes.
6. 15 mL Corning tubes.
7. Haemocytometer.
8. Trypan Blue Solution, 0.4 %.
9. Paraformaldehyde (PFA) 4 %.

2.1.2 Supplemented FAD-Medium Components

1. Adenine 100× aliquots:
   Prepare 0.05 N HCl by adding 0.205 mL 36 % HCl to 49.8 mL H_2O. Add 0.121 g adenine to 50 mL 0.05 N HCl. Stir solution for 1 h and filter through a 2 μm filter. Aliquot and store at −20 °C.
2. Hydrocortisone 1000× aliquots: Dilute 0.5 mg hydrocortisone per mL DMSO. Aliquot and store at −20 °C.
3. Cholera toxin 1000× aliquots: Make a 10^{-7} M concentration of Cholera Toxin in sterile water. Aliquot and store at −20 °C.
4. Penicillin/streptomycin solution (ThermoFisher Scientific).
5. GlutaMAX™.
6. human EGF.
7. Bovine insulin.
8. Chelated fetal bovine serum (FBS) aliquots:
   Heat-inactivate fetal bovine serum at 56 °C for 60 min. Add 200 g chelex 100 resin to 2 L of dH_2O and measure pH. Titrate the solution with HCl until pH = 7.4. Pour the solution through a filter paper and collect the chelex. Add the chelex to 500 mL serum and incubate overnight at 4 °C. Filter away chelex. Aliquot and store at −20 °C (*see* **Note 1**).
9. 100 mL FAD supplemented medium:
   67 mL DMEM no glutamine, no calcium, 22 mL Ham's F-12 Nutrient Mix, GlutaMAX™ Supplement, 10 mL chelated fetal bovine serum, 1 mL 100× adenine, 1 mL penicillin/streptomycin solution, 1 mL GlutaMAX™, 0.1 mL 1000× Cholera-Toxin, 0.1 mL 1000× hydrocortisone, 10 ng/mL human EGF, 5 μg/mL Bovine insulin (*see* **Note 2**).
   Sterilize by filtering the solution through a 2 μm filter. FAD supplemented medium can be stored at 4 °C for 10–14 days.
10. FAD starter medium:
    This cell medium is used for feeding the keratinocytes in the first 2 days in culture. Prepare 12 mL FAD supplemented medium per 6-well plate by adding Rock inhibitor to a final concentration of 10 mM.

2.2 Reagents for Flow Cytometry

(*see* Table 2).

Table 2
List of antibodies for analyzing and isolating known subpopulations by flow cytometry from telogen mouse epidermis

Antigen	Conjugate	Clone	Company	Concentration (per 10^6 cells)
Sca1	PE-Cy5	D7	BioLegend	0.08 µL
Lrig1	–	AF3688	R&D systems	10 µL
Plet1	–	33A10	Acris Antibodies	0.3 µL
Integrin α6	APC	GOH3	AbD Serotec	5 µL
CD34	Biotin	Ram34	eBioscience	5 µL
Streptavidin	PE	–	Life Technologies	5 µL
CD45	PE-Cy7	30-F11	BD Pharmingen	1 µL
CD31	PE-Cy7	390	BD Pharmingen	1 µL
CD200	PerCP-eFluor 710	OX90	eBioscience	0.25 µL
Integrin β1	PerCP-eFluor710	HMb1-1	eBioscience	0.25 µL
DAPI			Sigma	100 µg/mL

2.3 Characterization of Colonies

1. Crystal violet solution 1 %, aqueous solution.
2. Scanner.
3. Computer with Fiji image processing software (https://fiji.sc/).

3 Methods

Carry out all procedures at room temperature unless otherwise specified. The time line for experiments is indicated in Fig. 4a.

3.1 Preparation of 3T3 NIH Fibroblast Feeders for Keratinocytes Cultures

1. Expand 3T3 NIH-feeders in T75 tissue culture flasks seeding 2×10^5 cells. For a T75 use 14 mL of 3T3 NIH medium and change the medium every second day. 3T3 NIH feeders are grown at 37 °C in an atmosphere of 5 % CO_2 in a tissue culture incubator. Using these seeding densities, a T75 is confluent after 4–5 days and contains enough feeder cells to cover a 6-well plate.
2. 1 day before isolating keratinocytes, remove old medium from 3T3 cells. Mix 140 µL mitomycin C in 14 mL fresh 3T3 NIH medium and add the solution to the T75. Cells are subsequently placed at 37 °C in an atmosphere of 5 % CO_2 in a tissue culture incubator for 2 h to mitotically arrest the feeder cells.
3. Immediately after adding mitomycin to the feeder cells prepare the plates for the colony assays. Type I collagen is diluted

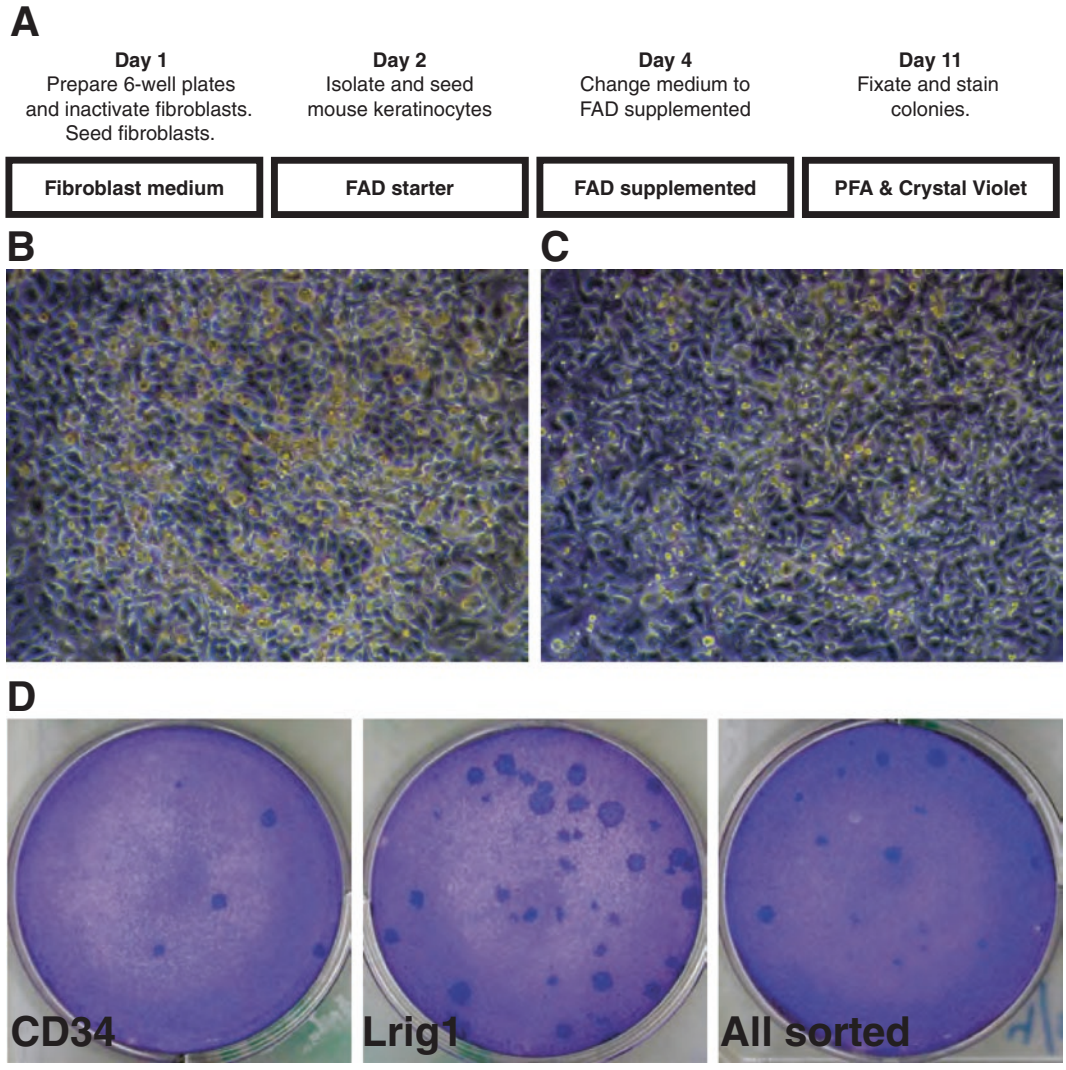


Fig. 4 Culturing epidermal keratinocytes. (**A**) Schematic flow chart showing the different steps in the protocol starting from seeding feeders to analyzing the clonal growth assays. (**B**, **C**) Examples of keratinocytes pushing away the feeders as an early indication of successful colony growth. (**D**) Examples of colony assays from epidermal keratinocytes that have been isolated based on either CD34 or Lrig1-eGFP expression. These colonies are stained with Crystal Violet. A white paper background is used to enhance the contrast

1/100 in sterile PBS and 1 mL is added to individual wells in a 6-well plate. Incubate at 37 °C for 2 h. Following the 2-h incubation rinse the plate once with PBS and add 2 mL PBS to each well.

4. After the 2-h incubation, remove the mitomycin-containing medium from 3T3 NIH feeder cells and rinse the T75 once with PBS. Trypsinize 3T3 NIH cells with 2 mL of 0.25 % Trypsin diluted in 8 mL Versene and wait until cells detach from the

plastic (after around 2 min). Transfer the solution to a 50 mL falcon tube. Pipet up and down to obtain a single cell suspension and add 15 mL 3T3 NIH medium supplemented with serum to 10 %. Centrifuge cells at 500 × *g* for 5 min and resuspend the pellet in 5 mL 3T3 NIH medium. Count the number of living cells per mL and dilute the suspension to 5×10^5 cells per mL in 3T3 NIH medium.

5. Remove PBS from the collagen-coated wells and add 2 mL of NIH cell suspension to each well and place the 6-well plates at 37 °C in an atmosphere of 5 % CO_2 in a tissue culture incubator.

3.2 Isolation of Keratinocytes from Mouse Back Skin

1. Euthanize the mouse by an approved technique such as cervical dislocation. The animal is subsequently clipped using a trimmer to remove as much hair as possible. It is an advantage to clip in the direction going against hair growth by moving the trimmer from tail to neck. The skin is subsequently sprayed with 70 % ethanol and dried with paper towel to remove loose hair (*see* **Note 3**).
2. In order to retrieve the back skin from a mouse, use forceps to grip the skin approximately 0.5 cm above the tail and cut through the skin with a scissor. Cut along the flanks from hind- to forelimbs until you reach the area behind the neck. With forceps pull the skin toward the neck and make a transverse cut along the neckline to remove the skin from the animal. Place the back skin in a petri dish and keep it on ice until further processing. The back skin can be kept on ice for several hours (*see* **Note 4**).
3. *Move the experiment to a sterile hood and use sterile equipment for the subsequent handling of the tissue (very important).*
4. Prepare a series of 50 mL falcon tube: one containing 30 mL diluted iodine, two with 70 % ethanol and one with PBS. Disinfect the tissue by incubating for 2 min in iodine, 1 min in each 70 % ethanol solution and wash in PBS for more than 30 s.
5. Place the tissue in a petri dish, with the dermal side facing upward. Use a scalpel to scrape of the adipose tissue from the dermal side using enough force to remove the membranous sheet attached to the skin without destroying the tissue (Fig. 5A) (*see* **Note 5**). Add 10 mL freshly prepared Trypsin 0.25 % to a new petri dish and place the scraped skin with the epidermal side up floating on the trypsin (Fig. 5B).Smooth out any wrinkles in the tissue with a forceps to allow ample access for the trypsin from the dermal side. Place the lid on the petri dish and incubate for 2 h at 37 °C in a cell culture incubator (*see* **Note 6**).
6. For each individual skin sample prepare two 50 mL falcon tubes and place a 70 μm cell strainer in one of them.
7. Transfer the skin to the lid of the petri dish, and scrape the epidermis off the dermis with a scalpel holding onto the skin with forceps (Fig. 5C). At this point you can dispose the dermis.

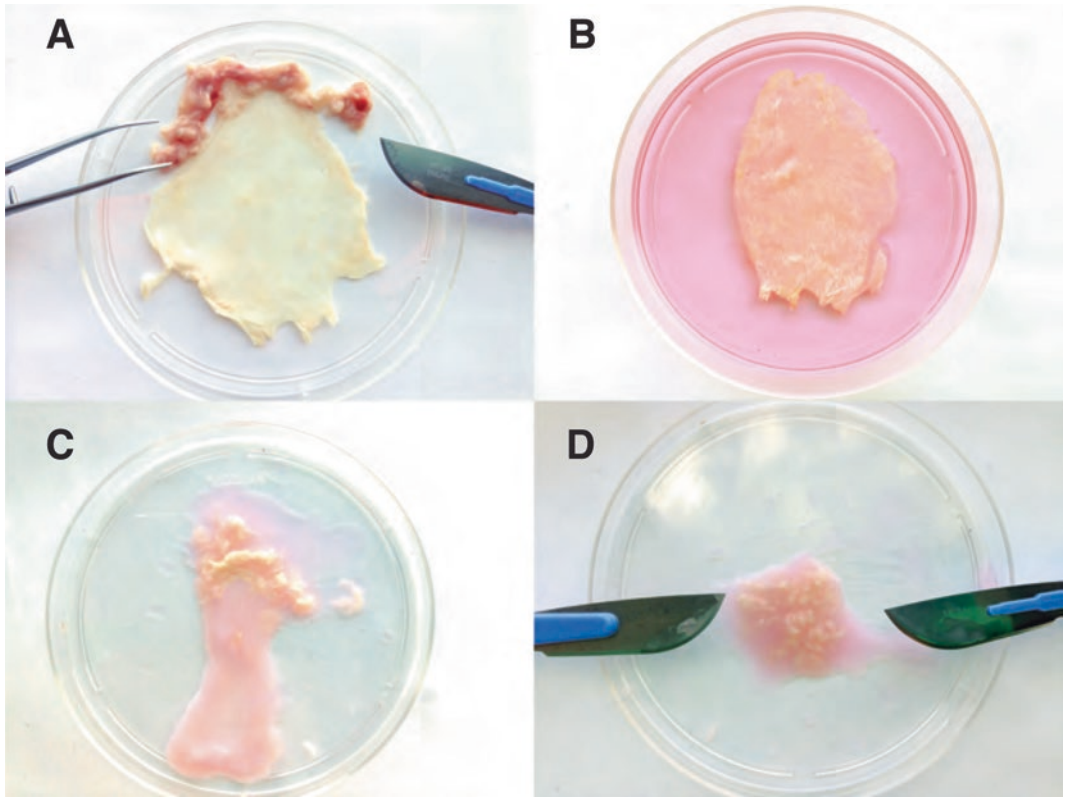


Fig. 5 Preparing mouse back skin for cell isolation. (**A**) Start by cleaning the dermal side of the skin by scraping off the adipose tissue and subcutis. (**B**) The clean mouse back skin is subsequently floated on trypsin with the dermal side facing the trypsin. (**C**) Following 2 h at 37 C the epidermis can be isolated from the dermis as a sheet or fragments using a scalpel to scrape the epidermal side of the tissue. (**D**) The epidermis is then minced vigorously with two scalpels

The epidermal sheets or fragments are cut into smaller pieces by cross cutting for 30–60 s with two scalpels (Fig. 5D) (*see* **Note 7**). The pieces need to be small enough to enter a 5 mL pipette for subsequent steps.

8. Transfer the minced tissue from the lid of the petri into a 50 mL falcon tube using the trypsin from the 2-h incubation. In order to break the tissue fragments further apart and release single cells into suspension, the solution is vigorously pipetted up and down 20–30 times using a pipette boy equipped with 5 mL pipette. The solution is then passed through the 70 μm cell strainer into a new 50 mL falcon tube (*see* **Note 8**). The solution should appear cloudy at this point as an indication for a good cell yield. Add 15 mL 4 °C 10 % ABS to deactivate the Trypsin. From this point and onward, it is important to keep the cells on ice or at 4 °C to reduce apoptosis.
9. Spin down the cells at 500 × *g* for 8 min in a cooled 4 °C centrifuge. Remove supernatant until there is approximately

10 mL left in the tube. Transfer the solution to a 15 mL corning tube and repeat the centrifugation at 500 × *g* at 4 °C for 8 min. A cell pellet should appear in the bottom of the tube. Remove the supernatant and resuspend the cells in 4 °C 5 mL PBS supplemented with 0.1 % BSA. Use a haemocytometer to determine cell numbers and Trypan blue to assess cell viability. A successful isolation will typically gain more than 10–20 million cells with minimal cell death (<5 %).

Skip steps 10 *and* 11 *if you are going to sort the keratinocytes by flow cytometry.*

10. Remove the medium from the 6-well plates, where the feeder cells were seeded on the previous day and add 2 mL FAD-starter medium to each well. The keratinocytes are subsequently added to the starter medium in the right seeding density (*see* **Note 9**). The number of keratinocytes seeded for clonal growth assays needs to be determined empirically, as colony-forming efficiency varies tremendously between strains. We typically seed 1000–3000 (back skin) and 3000–7000 (tail skin) keratinocytes per/well depending on mouse strain. Incubate the cells at 37 °C in a tissue culture incubator in an atmosphere of 5 % CO_2.
11. Change medium every 2 days (supplemented FAD-medium with no Rho-kinase-inhibitor). Analyze cultures after 10 days.

3.3 Antibody Labeling and Flow Cytometry of Epidermal Keratinocytes

Select an appropriate panel of antibodies for the target cells of interest (Table 2). When possible, select antibodies directly conjugated to fluorescent dyes. When this is not possible, different conjugation kits can be applied—i.e., SiteClick™ Qdot Antibody Labeling Kit (Life Technologies). Addition of a dye that enables exclusion of dead cells in the sort (i.e., DAPI) should be added a few minutes before cell sorting. The final antibody concentration of both experimental and compensation (single-stain) samples should be kept constant to ensure a precise correction of bleed-through prior to sorting, which is unavoidable, when performing multicolor flow cytometry. The suggested antibody concentrations in Table 2 are guidelines. Due to batch variation it is advisable to titrate new lots of antibody to optimize antibody concentrations.

1. Count cells and resuspend in sterile 14 mL tubes at a concentration of 5 × 10^6 cells per mL in staining buffer (PBS supplemented with 0.1–1 % BSA).
2. Aliquot the amount of cells necessary for each type of sample:
   (a) *Experimental sample(s)* for analysis and/or sorting—apply master-mix containing each antibody for the desired setup (Table 1) in the appropriate proportion (Table 2) at a final staining volume of 5 × 10^6 cells per ml.

(b) *Compensation samples* (one for each antibody used in the antibody master-mix as well as fluorescence minus one (FMO) controls). Optimally, use a minimum of 2.5×10^5 cells. Adjust to the staining concentration in the experimental samples (50 μL for 2.5×10^5 cells). Add only one antibody in each of these tubes. In addition, it is advisable to include FMO controls, where all antibodies except one are present. It is possible to perform incubations in Eppendorf tubes.

(c) *Unstained sample* Same setup as with the compensation samples but instead of adding antibody, add a dye to exclude dead cells (i.e., DAPI, 7AAD, or PI) in the same volume as in the experimental samples.

3. Incubate antibodies at concentrations according to guidelines and titration experiments for 30 min to 1 h in on ice, and protect the samples from direct light.
4. Spin down cells gently ($500 \times g$ for 3 min) and resuspend in 5 mL staining buffer—repeat this step twice.
5. If secondary antibodies or other conjugates are added, repeat **steps 4–6**, potentially with reduced incubation time (10–20 min for streptavidin).
6. Resuspend in staining buffer and transfer to FACS tubes or BSA-coated eppendorf tubes by filtering the cell suspension through a 70–100 μm mesh depending on the nozzle used for the subsequent flow cytometry. Remember to keep the samples on ice.
7. Add DAPI or an alternative nuclear stain prior to sorting.
8. Conduct sorting using either a 70 μm or 100 μm nozzle using the setup described in Fig. 3 for isolating CD34, ItgA6, and Lrig1 expressing cells. The example for Lrig1 expressing cells is based on a fluorescent reporter strain expressing GFP in Lrig1-positive cells.
9. Cells for seeding should be sorted into cold FAD complete medium and seeded shortly after the cell sorting is complete.
10. Seed cells into 2 mL FAD-starter-medium with Rho-kinase-inhibitor to each well.
11. Change medium every 2 days (supplemented FAD-medium with no Rho-kinase-inhibitor). Analyze cultures after 10 days.

3.4 Characterization and Quantification of Cell Cultures

1. After a few days you will begin to see the fibroblasts detach from the plate and get pushed aside by the keratinocyte colonies (Fig. 4b, c; *see*, **Note 10**).
2. After 10 days remove the medium and fix the cells in the dish by incubating in 4 % PFA for 15 min. Remove PFA, wash twice with PBS, and let the plate dry for 20 min. Add 2 mL of crystal

violet 1 % to each well for 20 min (*see* **Note 11**). Rinse in ample water and let the plate dry before quantification (Fig. 4d) (*see* **Note 12**).

3. Scan the plate at 600 DPI (*see* **Note 13**).
4. Measure the diameter of 1 well in mm.
5. Open the picture in Fiji (freeware: http://fiji.sc). Choose the *straight* tool from the tool bar.
6. Draw a line at the diameter of the well. From the menu bar choose *Analyze* → *Set Scale*. Type in the measured diameter of the well in "*Known distance*" and type "mm" in "*Unit of length*" and press *OK* (Fig. 6a).
7. From the menu bar select *Analyze* → *Set Measurements*. Check "*Area*" and "*Perimeter*."
8. Open ROI-manager from the menu bar at *Analyze* → *Tools* → *Roi Manager*. Select the option "*Show All*" and "*Labels*." Keep the *ROI manager* window open (Fig. 6c).
9. Select the *Oval*-tool from the toolbar (Fig. 6b). Draw a circle around a keratinocyte colony and press "*t*" on the keyboard. Repeat this step until you have marked every colony in the well. If you have very asymmetric colonies you should use alternative marking tools (*see* **Note 14**).
10. All the selected colonies are represented in the ROI-manager (Fig. 6d). Select one and go to the menu bar and select *Edit* → *Selection* → *Select All*. Press "*Measure*" in the ROI-manager. A results window appears that lists measurements of all the colonies. Select one and go to the menu bar and select *Edit* → *Selection* → *Select All*. In the menu bar press *Edit* → *Copy*. You can now paste the measurement data to Excel or other software for further analysis.
11. Delete all measurements in ROI-manager and results before quantifying colonies in the next well (*see* **Note 15**).
12. The area of the colonies can be presented in diagram showing the size of the individual colonies (Fig. 6e).
13. Colony-forming efficiency can be calculated as the percentage of keratinocytes seeded that formed a colony.

$$CFE = \frac{\text{Colonies}}{\text{Keratinocytes seeded}} \cdot 100\% \quad \text{(Fig. 6f)}.$$

4 Notes

1. We recommend to batch test several FBS lots due to variations in the composition. The lots should be tested in parallel to identify lots that support keratinocyte growth.
2. One experiment using a 6-well plate requires 60 mL FAD supplemented medium.

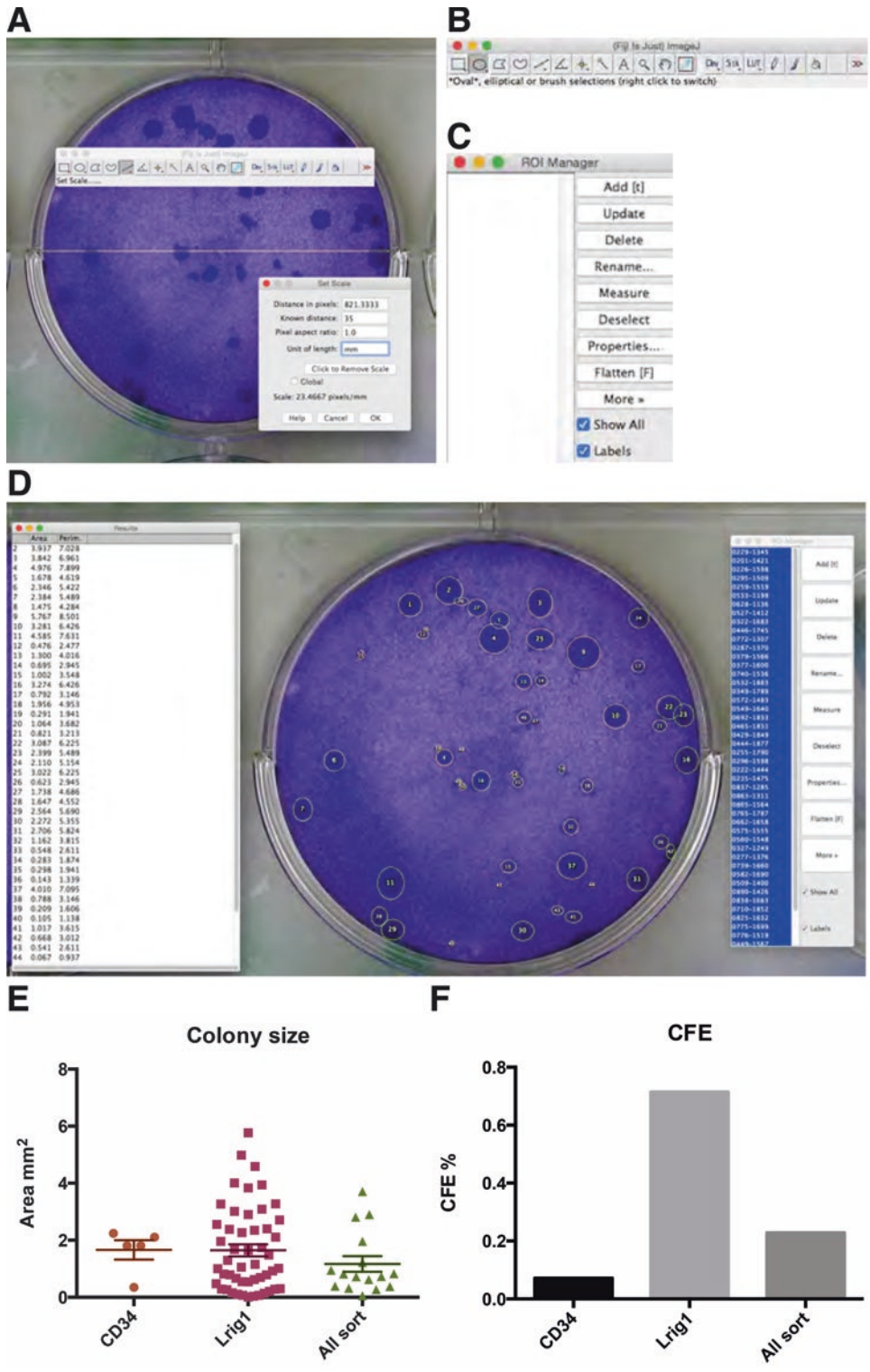


Fig. 6 Quantifying clonal growth assays. Scan plates from colony-forming assays at 600DPI and open the images in FIJI. (**a**) Choose the *straight* tool from the FIJI tool bar and calibrate the FIJI measurement system to a known distance. (**b**) Select the *Oval* tool from the toolbar. (**c**) Example of ROI-manager with no entries. (**d**) The workspace when measuring colonies, showing the measurements of all selections of colonies. (**e**) Representation of the colony area and (**f**) colony-forming efficiency for epidermal keratinocytes sorted based CD34 or Lrig1-eGFP. In these examples, an identical number of cells were seeded in the initial experiments and the data represents biological triplicates

3. All experiments using animals need to be approved by an appropriate ethical committee and a governing board in the country of residence.
4. In mice with pigmented hair, areas, which are dense in anagen hair follicles, will appear at a darker tone compared with catagen and telogen areas.
5. The skin on male mice is significantly thicker than that on female mice.
6. For optimal viability in tail skin preparations, use an incubation time of 1.5 h.
7. This is a crucial step that requires some practice. Moreover, trypsin can compromise cell viability and it is essential that this step is performed quickly and efficiently.
8. Remaining hairs and connective tissue can block the cell strainer. In order to avoid this lift the cell strainer or pipette gently onto the membrane to support the flow of cell suspension through the strainer.
9. Move the plate back and forth and side to side, and avoid that the solution swirl around the edge of the well as colonies will form here rather than being distributed throughout the well.
10. Macroscopic colonies can be spotted after 1 week, when looking from the bottom of the plate.
11. Crystal violet can be recollected, stored, and reused.
12. If you experience trouble with quantifying the colonies due to feeder background noise, it is possible to remove feeders. In this case, you need to wash the plate briefly in versene before fixation.
13. Cut round paper pieces of white paper that fits into the wells to get a better contrast in the scanned pictures.
14. You can save the selection overlays by choosing save in the ROI-manager.
15. Remember to paste data into another software program.

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Chapter 7

Endothelial Progenitor Cells: Procedure for Cell Isolation and Applications

Garikipati V.N. Srikanth and Raj Kishore

Abstract

Bone marrow endothelial progenitor cells (EPCs) have shown a great promise to promote ischemic tissue neovascularization and to attenuate ischemic injury in a variety of animal models, which led to EPC-based clinical trials that yielded modest but promising results. Some of the variables in the use of EPCs relate to their differential isolation and characterization protocols since the EPC literature does not identify a unique marker for these vascular progenitors. In this chapter, we present step-by-step protocols for the isolation of EPCs, their characterization and culture conditions, and their potential use in basic and clinical research.

Key words Endothelial, Progenitor cells, Bone marrow, Neo-vascularization, Angiogenesis

1 Introduction

For more than a decade now, biology and therapeutic efficacy of endothelial progenitor cells (EPCs) were largely driven by the first observations of Asahara et al. in identifying EPCs in adult peripheral blood (PB)[1] and were shown to derive from bone marrow (BM) further migrate and incorporate into foci of physiological or pathological neovascularization [2, 3]. As a matter of fact, postnatal neovascularization was believed to be established by the mechanism of "angiogenesis," by in situ proliferation and migration of preexisting endothelial cells (ECs) [4]. However, the finding that EPCs can home to sites of neovascularization and differentiate into ECs in situ is consistent with "vasculogenesis," a critical paradigm has been demonstrated in embryonic neovascularization [5] and also shown recently for the adult organism in which a pool of progenitor cells contributes to postnatal neovascularization [6]. The discovery of EPCs has therefore considerably changed our understanding of adult blood vessel formation. Furthermore, we and other groups envisage the potential of EPC to improve the clinical applicability in the fight against ischemic diseases.

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EPCs in circulation can be broadly subdivided mainly into two categories, hematopoietic lineage EPCs and nonhematopoietic lineage EPCs. The hematopoietic EPCs originate from BM and represent a pro-vasculogenic subpopulation of hematopoietic stem cells (HSCs) [7, 8]. The non-hematopoietic EPCs are blood or tissue-derived entities, exhibiting EC like phenotype [9] or ability to differentiate into EC like cells [10]. Hematopoietic EPCs (hEPCs) have been shown to express a variety of cell surface markers, including membrane receptors like CD31, CD133, (Fetal liver kinase-1) Flk-1, CXC chemokine receptor-4 (CXCR-4), CD105 (Endoglin), c-Kit for human samples and receptors like c-Kit (CD117), stem cell antigen (Sca-1), and CD34 in combination with Flk-1 (vascular endothelial growth factor receptor-2 (VEGFR-2)) in case of mouse samples [11]. Nevertheless, identifying a particular combination of markers for isolation of these cells has been a big challenge for the researchers in this field.

Our methodology utilizes the conventional EPC culture methods to produce spindle adherent cells from BM and the same protocol can be extrapolated to the peripheral blood (PB), umbilical cord mononuclear cells (UCBMNCs) with endothelial growth factors and cytokines. These assays using conventional EPC culture protocols are simple and satisfactory to speculate the vasculogenic nature of the EPC-enriched fractions. We have successfully used these cells to understand various signaling pathways involved in EPC-based cardiac repair and regeneration [12–17]. In this chapter, we present a step-by-step protocol for the isolation of EPCs and their culture.

2 Materials for Endothelial Progenitor Cells Isolation and Culture

1. DPBSE (*Ca^{2+}; Mg^{2+}free*).
2. 0.5 % mM EDTA.
3. Histopaque 1083.
4. Ammonium Chloride (NH_4Cl).
5. Human fibronectin.
6. EBM-2 Bullet kit.
7. EBM-2 Basal Medium 500 mL.
8. EGM-2 Single Quot Kit Suppl. & Growth Factors: LONZA (Note: do NOT add Hydrocortisone to the media).
9. 6 well plates.
10. 10 mL pipettes.
11. 5 mL pipettes.
12. Screw cap sampling tubes (15 mL).
13. Screw cap sampling tubes (50 mL).

14. Cell Strainers.
15. BD-20 mL Syringe.
16. Pestle and Mortar.

3 Basic Protocol-EPC Isolation from Mouse Bone Marrow

1. Take as much blood as possible from one mouse.
2. Dissect all bones with muscle roughly and soak them in DPBSE (PBS *without* Ca^{2+} *and* Mg^{2+},5 mM EDTA).
3. Remove muscle (spinal cord) from bones including spine, soak the cleaned bones in DPBSE.
4. Transfer all bones to mortar and cut with appropriate scissors.
5. Gently tap and squeeze cells out of bone using pestle, do this in DPBSE (*use 10 mL for first time and 5–7 mL each subsequent time*).
6. Collect supernatant (cell solution) with 18G syringe (10 mL) to a 50 mL tube with 70 μm cell strainer.
7. Repeat **step 6**, 3 times until the supernatant becomes clear and bone fragments are white (gradually apply more pressure to the bone fragments each time).
8. Collected cell solution should be 40 mL, or just increase the volume to 40 mL with DPBSE.
9. Coat the inside of a new 50 mL tube with 10 mL *ROOM TEMPERATURE* Histopaque 1083 *SLOWLY* add the 40 mL of cell solution to the tube with Histopaque 1083, keeping the two layers separate (50 mL in total, if not enough add DPBSE).
10. Centrifuge (Cfg). 2150 rpm (430 × *g*), 20 min, RT without brake (#1, select then turn the knob).
11. Aspirate down to 15 mL, collect MNC layer (buffy coat) to new 15 mL tube with 18G syringe (3 mL).
12. Add DPBSE to 14 mL and invert tube to mix.
13. Cfg. 2450 rpm (1000 × *g*), 5 min, 4 °C with low brake.
14. Aspirate to 1 mL and pipette to dissolve cell pellet.
15. Add DPBSE up to 14 mL and Cfg. 1300 rpm (280 × *g*), 5 min, 4 °C with low brake.
16. Aspirate to 1 mL and pipette to dissolve cell pellet.
17. Add 6 mL of NH_4Cl Cfg. @1400 rpm (320 × *g*), 5 min, 4 °C.
18. Aspirate NH_4Cl, then add DPBSE up to 14 mL and Cfg. 1400 rpm, 5 min, 4 °C with low brake (level 5).
19. Coat appropriate plates/dishes with human fibronectin (5 μg/mL) for 1 h at 37 °C.

20. Aspirate the supernatant and add EBM2 containing EGM2-MV Bullet kit medium (10 % FBS without hydrocortisone) up to 4 mL (for each mouse) to 10 cm^2 dish without coating, incubated in 37 °C for 30–40 min.
21. Transfer the supernatant to 6-well plate coated with 5 μg/mL human fibronectin (1 mL/per well).
22. Count cells.
23. Plate the cells appropriately.

4 Applications

The above protocol provides the methodology to isolate EPCs for research into basic or clinical EPC-based therapeutics. The applications of the same include.

4.1 Basic Research

1. Evaluation of the effect of target factors on EPC expansion and/or differentiation like growth factors cytokines, hormones, cell signaling regulators, etc.
2. Delineate EPC differentiation signaling pathways.
3. Cell fate of HSC in hematopoiesis and vasculogenesis.

4.2 Clinical Research

1. Evaluation of pathophysiology in cardiovascular diseases, in terms of EPC biology.
2. Evaluation of vascular regenerative potential of EPCs in cardiovascular diseases.

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Chapter 8

Therapeutic Application of Placental Mesenchymal Stem Cells Reprogrammed Neurospheres in Spinal Cord Injury of SCID

Vikram Sabapathy, Franklin Jebaraj Herbert, and Sanjay Kumar

Abstract

Mesenchymal stromal cells (MSCs) and induced pluripotent stem cells (iPSCs) have stimulated much interest in the scientific community and hopes among the general public since their discovery in 1966 due to a variety of potential applications it has in the field of regenerative medicine. Copious amount of literature, as well as long-term animal and human clinical trials, indicates that MSCs can be successfully used for therapeutic purpose without any extreme adversities. MSCs have been isolated from adult and fetal tissues. Recently, MSCs from placenta have generated much inquisitiveness. In this article, we will demonstrate the step-by-step procedure for isolating human placental MSCs from term placenta, reprogramming of placental MSCs into iPSCs using plasmid vectors, evaluation of functional recovery in mice spinal cord injury models, and in vivo tracking of the transplanted cells.

Key words Mesenchymal stem cells, Induced pluripotent stem cells, Reprogramming, Neurospheres, Spinal cord injury, Indocyanine green

1 Introduction

Spinal cord injury is a debilitating disorder that results in loss of motor/sensory neuronal function due to damage to the spinal cord [1]. At present, there is no formal treatment available for curing spinal cord injury; consequently, alternate cell therapy-based methods are tested for treating the spinal cord injury. Recent studies have positively reported various cell therapeutic strategies for treating spinal cord injury [1].

Since their discovery in 1966 MSCs have evoked many interests in therapeutic applications [2]. MSCs are a class of stem cells that self-renew and differentiate into cells of multiple germ layers. MSCs have been isolated from an adult as well as fetal tissue sources. Immunomodulation is one of the main important features of MSCs. Off late, the source of origin of MSCs is gaining much attention due to enhanced characteristic properties of the MSCs

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from fetal tissues than adult tissue sources [3, 4]. Reports from previous pre-clinical experiments have demonstrated that employment of MSCs resulted in systematic functional recovery from spinal cord injury following reduced demyelination, suppression of inflammation, and promotion of neuroprotective molecules [1]. Among the fetal tissue sources, placenta serves as an abundant and reliable source of MSCs. The term placenta is considered a medical waste and discarded after surgery. In this study, placental MSCs were obtained from the cotyledon region and subjected to orderly phenotypic and genotypic characterizations.

In 2006 and 2007, Yamanaka and colleagues demonstrated the ability to develop cells with embryonic stem cells like property called iPSCs from mouse and human fibroblasts respectively with the help of defined transcription factors [5, 6]. Over the course of time, many labs around the world have successfully reproduced the technique and subsequently improved the safety and efficacy of generation of iPSCs using various viral, non-viral, and small molecules strategy [7]. In this protocol, we describe the efficient generation of safer, non-integration, and virus-free iPSCs using nucleofection and small molecules. Characterization of the generated iPSCs cell lines was carried out using immunostaining, qPCR, epigenetic analysis, in vitro differentiation assay, embryoid body formation, and microarray analysis. The neurospheres generated from human iPSCs were transplanted into SCID mice spinal cord injury models and accessed for functional recovery. After transplantation, the actual fate of the transplanted cells in vivo is not known. Hence, we have used the novel indocyanine green (ICG) based cell labeling technique to track the transplanted cells [8].

This protocol delineates the necessary procedure to be followed to study the spinal cord injury regeneration using SCID mice models.

2 Materials

All chemical and reagents were purchased from Sigma®, unless or otherwise mentioned.

2.1 Isolation of Human Placental MSCs

1. Plastic bag (Ethylene oxide sterilized).
2. Sterilized tray.
3. 1× Phosphate buffer saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4.
4. Antibiotic solution: Penicillin (100 units/mL)-Streptomycin (100 µg/mL) (Gibco), Gentamycin (5 g/mL), Amphotericin B (2.5 g/mL).
5. 0.25 % Trypsin-EDTA.

6. 0.05 % Trypsin-EDTA.
7. 250 μm metal sieve.
8. Collagenase I (12.5 U/mL).
9. 100 μm cell strainer.
10. Red blood cells (RBC) lysis buffer: 0.1 mM EDTA (pH 8), 155 mM NH_4Cl, 12 mM $NaHCO_3$.
11. MSCs expansion medium: α-MEM with ribonucleosides and deoxyribonucleosides (Lonza), 1 mM L-Glutamine (Gibco), 10 % Fetal Bovine Serum (Gibco), 1 % Penstrep (Invitrogen).
12. Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium (Lonza).
13. CO_2 humidified incubator.

2.2 Characterization of Placenta-derived MSCs

1. Trypan Blue (0.5 % in PBS).
2. Flow-Cytometer Antibodies: CD 14, CD34, CD45, CD29, CD73, CD90, CD105, HLA-DR, and mouse IgG1k controls (e-biosciences, California, USA).
3. Adipogenesis differentiation Kit (STEM PRO—Life Technologies, Thermo Scientific, USA).
4. Osteogenesis differentiation Kit (STEM PRO—Life Technologies, Thermo Scientific, USA).
5. Chondrogenesis differentiation Kit (STEM PRO—Life Technologies, Thermo Scientific, USA).
6. Oil Red O.
7. Methanol.
8. 5 % Silver Nitrate solution.
9. 5 % Sodium Thiosulphate.
10. 10 % Formalin.
11. 3 % Alcian Blue.
12. 0.1 % Safranin O.
13. Xylene.
14. Ethanol.
15. DPX Mounting media.
16. 3 μM 5-azacytidine.
17. 5 μg/mL Hoechst 33342.
18. 5 mM ß-Mercaptoethanol.
19. Neural differentiation antibodies: NeuN, Map2, GFAP, Neurofilament (Cell Signalling Technologies, USA).
20. Taurine (50 μM).
21. 0.1 % Gelatin.

22. 10 mM Nicotinamide.
23. Pancreatic differentiation antibodies: PDX1, Insulin (Cell Signalling Technologies, USA).
24. 0.05 % Colchicine.
25. Triton X-100.
26. PBST: 1× PBS, 0.1 % Tween 20.
27. 1 % BSA.
28. Trizol (Invitrogen—Life Technologies).
29. DyNamo HS SYBR® Premix (Finnzymes, Thermo Scientific, USA).
30. SuperScript® III First-Strand Synthesis SuperMix (Life Technologies).
31. Agar-Agar.
32. 50 μg/mL Propidium Iodide.
33. 10 μg/mL RNase A.
34. Apoptosis Kit—7AAD Annexin V (BD Pharmingen).

2.3 Derivation and Characterization of iPSCs

1. Nucleofection Kit (Lonza).
2. Plasmids: AAV OSKM, AAV OSK, AAV OSNK, AAV OSNL.
3. iPSCs media.
4. iPSCs expansion media.
5. DMEM/F-12 media (Invitrogen).
6. 20 % KOSR (Invitrogen).
7. 5 ng/mL βFGF (Invitrogen).
8. 50 U/mL Penicillin (Invitrogen).
9. 50 μg/mL streptomycin (Invitrogen).
10. 0.1 mM β mercaptoethanol.
11. 1 mM L-Glutamine (Invitrogen).
12. 100 mM Nonessential Amino Acids (Invitrogen).
13. GSK inhibitor—Chir99021 (3 μM).
14. MEK inhibitor—PDO325901 (1 μM).
15. Human LIF—(10 ng/mL).
16. mTeSR media.
17. Rock inhibitor Y27632 (10 μM).
18. Dispase (2 mg/mL).
19. iPSCs Antibodies: OCT4A, SOX2, Nanog, SSEA4, Tra 1-81, Nestin, SMA, AFP, CD71 (Cell Signalling Technologies, USA), Stain Alive Tra 1-81 (Stemgent).
20. Stem Diff: Neural induction media (Stem Cell Technologies).
21. PLO (10 μg/mL)/Laminin (1 μg/mL).

22. Matrigel (BD Pharmingen).
23. E1: DMEM with 20 % FBS, 1 % nonessential amino acids (NEAA), 1 mM L-glutamine and 0.1 mM β-mercaptoethanol (MTG), supplemented with 40 ng/mL bone morphogenetic protein 4 (BMP4), and 40 ng/mL vascular endothelial growth factor (VEGF).
24. E2: X-VIVO 15 (Lonza) with 1 % NEAA, 1 mM L-glutamine, and 0.1 mM MTG supplemented with 40 ng/mL BMP4, 40 ng/mL VEGF, 20 ng/mL FGF2, 40 ng/mL stem cell factor (SCF), 40 ng/mL Flt3 ligand (Flt3L), and 40 ng/mL thrombopoietin (TPO).
25. Accutase.
26. E3: X-VIVO 15 supplemented with 20 % BIT (Stem Cell Technologies), 1 % NEAA, 1 mM L-glutamine, 0.1 mM MTG, 100 ng/mL SCF, 6 U/mL erythropoietin, and 10 μM dexamethasone.
27. Giemsa stain.
28. P1 Media: DMEM/F12 media, 1 mM L-glutamine, 1 % ITS, 1 μg/mL Fibronectin, 10 μM Retinoic Acid, 100 ng/mL Activin A.
29. P2 Media: N2B27 Media, 10 ng/mL ßFGF.
30. P3 Media: N2B27 Media, 10 ng/mL ßFGF, 10 mM Nicotinamide.
31. Non-Denaturing Lysis Buffer: 1 % Triton X-100, 10 % Glycerol, 50 mM Tris, pH 7.4, 150 mM Sodium Chloride, 1 mM PMSF, 2 mM $MgCl_2$, 1 mM DTT, 2 mM EGTA.
32. TBST: 20 mM Tris base, 137 mM NaCl; pH 7.6 0.1 % Tween-20 with 3 % BSA.
33. PVDF membrane (Amersham).
34. ECL prime (Amersham).
35. Stripping Buffer: 50 mM Tris Base pH 6.8, 2 % SDS, 100 mM ß-mercaptoethanol.
36. 3 M NaOH.
37. Bisulfite/hydroquinone solution.
38. 3 M Sodium Acetate.
39. Glycogen.
40. Isopropanol.
41. 70 % ethanol.
42. TE (Tris–EDTA) buffer.
43. 3 M NaOH.
44. pCR 2.1-TOPO vector (Invitrogen).
45. Contig Alignment, Vector NTI (Invitrogen).

46. Protease inhibitor cocktail (Roche).
47. Phosphatase inhibitor cocktail (Roche).
48. PVDF membrane (Amersham)
49. 12 % SDS poly-acryamide gel.
50. Western Blot antibodies: P-Cadherin, Nanog, B-Actin.
51. Hematoxylin and esoin.
52. Tris–EDTA (TE) Buffer: Tris–HCl (10 mM, pH 8), EDTA (1 mM, pH 8).
53. Human gene expression 8x60K chip (Agilent technologies).
54. Mitomycin C (10 μg/mL).
55. Gelatin.

2.4 Animals

2.4.1 Materials Required

1. Adult Male/Female SCID mice (B6.CB17-prkdcScid/SzJ).
2. Ketamine-Xylazine cocktail.
3. Ringer lactate (Baxter).
4. Ciprofloxacin.
5. Meloxicam.
6. Normal Saline (0.9 %) (Baxter).
7. ICG (Sigma-Aldrich Co., St. Louis, MO).

2.4.2 Surgery

1. Hamilton/BD insulin syringe.
2. 3D stabilizer.
3. Suture-Vicryl.

2.5 Anesthesia System

1. Isoflurane vaporizer
2. Supply gas (oxygen)
3. Supply gas regulator
4. Flowmeter
5. Induction chamber
6. Connection tubing and valves
7. Facemask or intubation supplies
8. Scavenging method (Active/Passive)

2.6 Surgical Tools

1. Straight sharp scissors.
2. Steel container.
3. Steel tray.
4. Forceps (Dumont #5 - Switzerland).
5. Surgical blade.
6. Surgical gloves.

2.7 Consumables

1. Centrifuge tubes 15 and 50 mL.
2. Pipettes—5, 10, and 25 mL.
3. Tissue culture flasks: T25, T75 flask.
4. FACS tubes.
5. Cell culture plates: 24 well.
6. Cell culture dishes: ultra low attachment dish.
7. 22G needle.

2.8 Instruments

1. Shaking water bath.
2. Centrifuge.
3. Biosafety cabinet—Level 2.
4. CO_2 incubator.
5. Flow cytometer.
6. Inverted Microscope (Leica, Wetzlar, Germany).
7. Improved Neubauer Haemocytometer.
8. Microtome (Leica, Germany).
9. Light microscope (Leica, Germany).
10. Fluorescent microscope (Leica, Germany).
11. Axioplan microscope (Zeiss, Germany).
12. 12K Flex Quant Studio (Life Technologies, USA).
13. Thermocycler (Applied Biosystems, USA).
14. Nucleofector (Lonza).
15. FluroChem E Imaging system (Protein Simple).
16. Heating pad.
17. Infra-Red Lamp.
18. IVIS imaging station (Perkin Elmer, MA, USA).
19. BSC Level 2 (Thermo Scientific).

3 Methods

3.1 Collection and Isolation of Human Placental Derived MSCs

1. Before the collection of the placental samples ethical and technical consent is obtained from the ethical committee of the institution.
2. After obtaining the written consent from the patient undergoing elective caesarean, collect the term placenta obtained from a caesarean section in an ethylene oxide sterilizer plastic bag and transport immediately to the tissue-processing lab (Fig. 1i) (*see* **Note 1**).
3. Carefully transfer the placental sample onto sterilized tray (Fig. 1ii, iii) (*see* **Note 2**).

4. Wash the placental tissue using 1× PBS with antibiotics and discard the solution. Repeat the washing for three times with 1× PBS with an antibiotic solution (Fig. 1iv–vi) (*see* **Note 3**).
5. Cut open the placental membrane and remove the blood clots from the surface (Fig. 1vii).
6. About 80 g of placental cotyledon tissue of uniform size is cut, and the remaining placenta is discarded into the biological waste disposable bag (Fig. 1viii).
7. Now, wash the placental tissue using 1× PBS. Repeat the washing for three times.
8. Chop the tissue sample into small pieces with the help of forceps and scissors while simultaneously removing the clots (Fig. 1ix) (*see* **Note 4**).
9. Now wash the small tissue samples in 1× PBS for three times (Fig. 1x–xii).
10. Weigh exactly 60 g of tissue sample and transfer 15 g of tissue sample into four 50 mL falcon tubes (Fig.1xiii).
11. Add 25 mL of 0.25 % Trypsin-EDTA in each centrifuge tube and incubate the samples at 37°C in a shaking water bath for 90 min[3] (Fig. 1xiv, xv) (*see* **Note 5**).
12. Post incubation, subject the tissue samples to a 250 μm metal sieve. Collect the retentate tissue sample from the sieve and equally distribute it into four 50 mL centrifuge tubes (Fig. 1xvi, xvii).
13. Add 25 mL of 12.5 U/mL collagenase I into each of the centrifuge tubes and incubate the sample at 37°C shaking water bath for 90 min (Fig. 1xviii).
14. After digestion, allow the sample to pass through 250 μm metal sieve and collect the filtrate (Fig. 1xix–xxi). Now, pass the filtrate through 100 μm cell strainer and collect the enriched filtrate.
15. Transfer the filtrate into centrifuge tubes. Centrifuge at 650 × *g* for 10 min and discard the supernatant and resuspend the pellet in 5 mL of RBC lysis buffer (Fig. 1xxii, xxiii).
16. Place the samples on ice for 20 min with occasional invert mixing of the sample (Fig. 1xxiv).
17. Centrifugation the sample at 1500 RPM for 10 min (Fig.1xxv).
18. Previous two steps can be repeated once more if RBC lysis does not happen properly (Fig. 1xvi, xvii) (*see* **Note 6**).
19. Count the number of cells using a hemocytometer. Seed 1×10^6 cells per T75 Flask containing 25 mL of MSCs expansion medium (Fig. 1xviii, xxix).
20. Place the flasks in a 37°C CO_2 incubator and check for a change in media color (pH and nutrients) or contamination once in 2 days (Fig. 1xxx). Change the media every 3 days once.

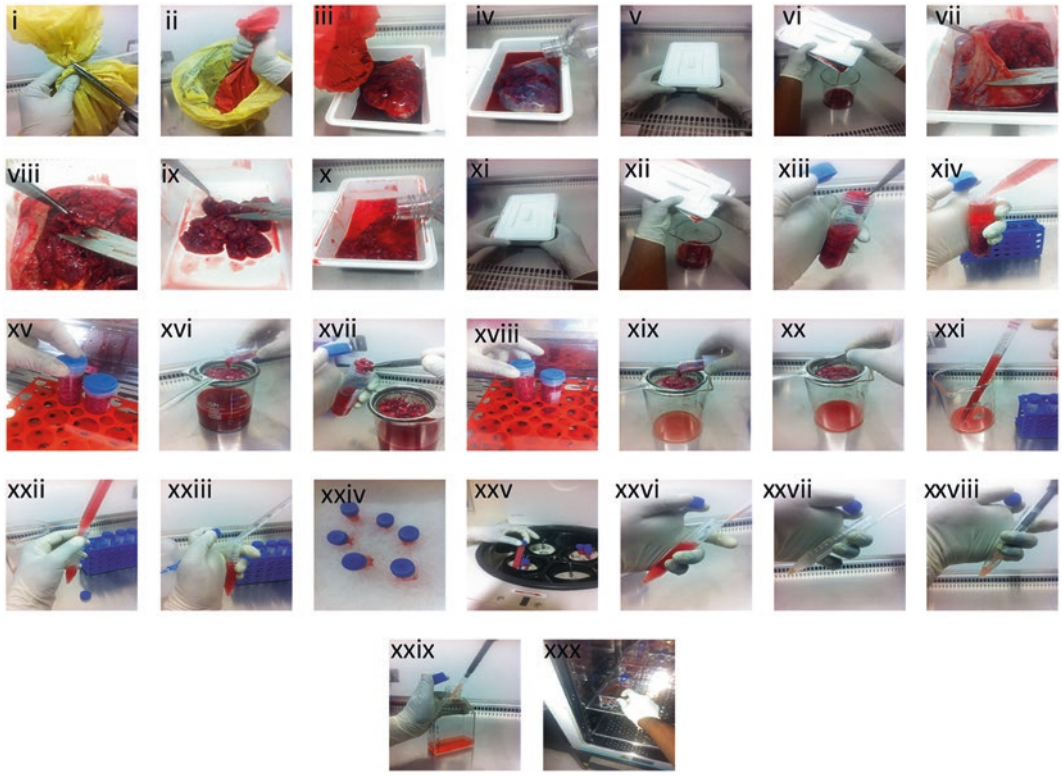

Fig. 1 Overview of isolation of placental MSCs. **i** - Term placenta obtained by caesarean section collected in sterilie plastic bag. **ii-iii** - 2 layer bag unopened and sample transferred onto sterilie tray. **iv-vi** - Placental tissue washed with PBS. **vii** - The placental membrane cut-opened and blood clots removed from the surface. **viii** - Placental cotyledon tissue cut uniformly. **ix** - Placental cotyleden tissue chopped into small pieces while simultaneously removing clots. **x–xii** - The small tissue pieces washed with PBS. **xiii** - Tissue samples weighed and transferred to 50ml falcon tubes. **xiv-xv** - Trypsin-EDTA added and incubated in shaking water bath. **xvi-xvii** - Retentate tissue sample distributed into centrifuge tubes post subjection through a 250 m metal sieve. **xviii** - Addition of collagenase I followed by incubation in shaking water bath. **xix-xxi** - Sample subjected through 250m metal sieve and the filtrate, which inturn, passed through 100m cell strainer to collect the enriched filtrate. **xxii-xxiii** - The filtrate transferred into 15ml centrifuge tubes and pelleted by centrifugation followed by resuspension in RBC lysis buffer. **xxiv** - Samples placed on ice with occasional invert mixing. **xxv-xxvii** - Centrifugation followed by repetition of the RBC lysis if required. **xxviii-xxix** - Cells counted using hemocytometer and seeded in T75 Flask containing MSC expansion medium. **xxx** - Seeded flasks placed in CO_2 incubator and monitored regularly

21. At the end of the 14th day, confluent monolayer cells can be observed (Fig. 2).
22. On the 14th day, passage the cells, 1:2 ratio into the T75 flask. The flasks should be confluent in 3–4 days.
23. A good practice would be to check the level of confluency every day and split the cells into 1:2 ratio as soon as the cell confluency reaches around 80 % (*see* **Note 7**).

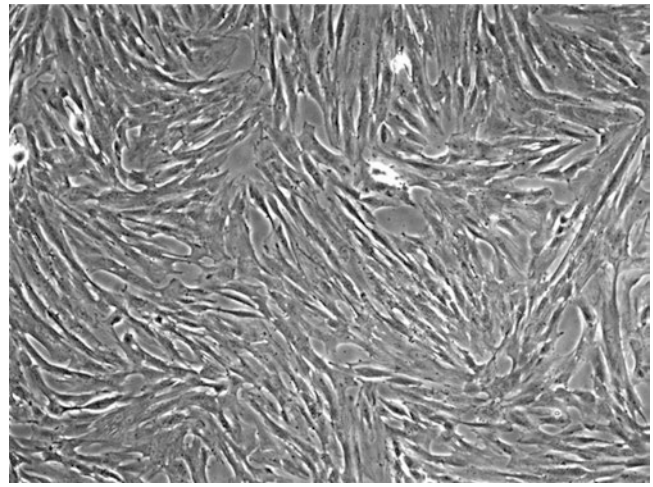

Fig. 2 Confluent monolayer culture of human placenta derives MSCs

3.2 Characterization of the Perinatal Placental MSCs

3.2.1 Flow Cytometer-Based Surface Marker Analysis

1. Trypsinize the cells with 0.05 % Trypsin-EDTA after a wash with DPBS.
2. Equally aliquot (1×10^5 cells per reaction) into FACS tubes (*see* **Note 8**).
3. The Unstained antibody and cells stained with isotype antibody act as controls; Stain the cells with the respective antibody.
4. Add the antibodies to the cells in the dark to avoid bleaching.
5. Incubate the samples at room temperature in the dark for 20 min.
6. Wash the cells with 1 mL of DPBS and centrifuge at 650 × *g* RPM for 5 min.
7. Resuspend the pelleted cells in 300 μL DPBS and analyze with a flow cytometer (FACS Calibur; Becton Dickinson) (*see* **Note 9**).
8. Acquire a Minimum of 10^4 gated events for each sample for analysis using Quest Cell Pro (Becton Dickinson).

3.2.2 Differentiation Analysis

1. Adipocyte differentiation
   (a) Ensure that the monolayer is in the mid-log growth phase and the confluence is approx 60–80 % (*see* **Note 10**).
   (b) Aspirate the growth medium and the debris/floating cells from culture flask and wash with PBS.
   (c) Add 0.05 % Trypsin to the flask and ensure that the culture surface is completely covered.
   (d) Incubate for 2–3 min at 37°C or until cells have detached completely.
   (e) Add FBS containing media to inactivate the trypsin, pipet the isolated cells gently to ensure a single cell suspension, and verify on an inverted microscope.

(f) Transfer into a 15 mL centrifuge tube, and pellet the cells at 1000 RPM for 5 min.

(g) Determine the cell viability and cell density using trypan blue and hemocytometer.

(h) Resuspend the cells in prewarmed MSC expansion medium and seed onto wells of a 24-well plate at a density of 5×10^4 cells/well.

(i) Incubate in a CO_2 humidified incubator at 37°C for 3–4 days.

(j) Replenish the media with 1 mL of prewarmed complete adipogenesis differentiation media.

(k) Replenish with fresh differentiation media every 48 h.

(l) After a period of 30 days, remove the media and process for classical staining with Oil Red O and other gene expression analysis/protein expression.

2. Osteocyte Differentiation
Trypsinize the monolayer and suspend in PBS as described in Subheading 3.2.2.1 (**steps a–g**)

(a) Seed MSC's at a concentration of 5×10^4 cells in a 24-well plate containing osteogenic induction medium (STEM PRO—Invitrogen) (*see* **Note 11**). Note: Make sure the cells are seeded at an optimal concentration low enough to compensate for the rapid proliferation in 30 days and high enough to ensure communication and to prevent senescence.

(b) Replenish with freshly warmed induction media every 48 h for 30 days.

(c) After 30 days, carefully remove the media and fix the cells directly with 1–2 mL of methanol pre-cooled at −20 °C.

(d) Incubate the plate at −20 °C for 20 min.

(e) Remove the methanol and wash with DPBS.

(f) Treat the well with 5 % Silver Nitrate solution, and expose to UV light for 1 h under the laminar hood.

(g) Wash the excess $AgNO_3$ with water and add 5 % Sodium Thiosulphate and incubate for 2 min at room temperature.

(h) Rinse with sterile water and observe under the light microscope for the presence of extracellular calcium.

3. Chondrocyte differentiation

(a) Trypsinize the monolayer and suspend in PBS as described in Subheading 3.2.2.1 (**steps a–g**).

(b) Pellet 1×10^6 MSCs at $650 \times g$ in a 15 mL Falcon Tube (*see* **Note 12**).

(c) Add Chondrocyte differentiation medium without disturbing the pellet.

(d) Change media every 48 h for a total period of 30 days.

(e) Ensure that the original media is slightly warmed and add the media very gently down the sides to make sure the pellet is not disturbed (*see* **Note 13**).

(f) After the differentiation period, fix the pellet with 10 % formalin for 1–2 h.

(g) Stain the pellet with merchrome and embedded in paraffin wax.

(h) Make sections of 5 μm in the microtome (Leica—Germany) and deparaffinize with xylene and hydrate with gradient ethanol wash.

(i) Flood the individual slides with Safranin O and 3 % Alcian blue for proteoglycans.

(j) Rinse the slides with distilled water.

(k) Air-dry the slides at room temperature.

(l) Immerse in xylene.

(m) Mount with DPX and allow for air-drying completely and observing under a microscope.

4. Tubular assay

(a) Trypsinize the monolayer and suspend in PBS as described in Subheading 3.2.2.1 (**steps a–g**).

(b) Seed 5×10^4 placental MSCs in a 25 cm^2 flask containing 5 mL Mesenchymal expansion medium with 3 μM 5-azacytidine.

(c) Culture the cells for 21 days with media changes every 7 days.

(d) Stain the cells with Hoechst 33342 and incubate at 37°C for 30 min before observing under the fluorescent microscope.

5. Neural

(a) Seed 5×10^5 placental MSCs into a T75 flask with serum-free α-MEM containing 5 mM β-mercaptoethanol.

(b) Culture the cells for 6–9 h at 37°C in a humidified CO_2 incubator with 5 % CO_2.

(c) Remove the media, wash with PBS and fix the cells with methanol as described earlier in Subheading 3.2.2.2 (**steps c–e**).

(d) Wash with PBS and proceed with Immunostaining with the specific neural marker NeuN, Map2, GFAP, Neurofilament.

6. Retinal
   (a) Seed 1 × 10^5 Placental MSC's into a 12-well plate with mesenchymal expansion medium supplemented with 50 μM Taurine with 1 mM Beta-mercaptoethanol.
   (b) Culture the cells for 8 days. Replenish with freshly warmed media every alternate day.
   (c) After 8 days, remove the media, wash with PBS and collect the cells in Trizol, pipette vigorously, and incubate for 5 min at room temperature to completely dissociate the nucleoprotein complex.
   (d) Freeze immediately at −70 °C or process the sample for RNA extraction, cDNA conversion, and qPCR using manufacturer's protocols.
   (e) Process the other well by fixing with cold methanol and proceed with immunocytochemistry as described earlier.
7. Pancreatic
   (a) Treat two 25 cm^2 flasks with Gelatin and leave at room temperature for 2 h.
   (b) Seed 5 × 10^5 cells onto the gelatinized dish containing mesenchymal expansion medium with 10 mM nicotinamide and 1 mM β-Mercaptoethanol and leave for 24 h for preinduction.
   (c) Treat the cells with mesenchymal expansion medium without FBS but containing 10 mM Nicotinamide and 1 mM β-Mercaptoethanol for 6 h, and for the next 18 h, treat the cells with the induction media containing FBS.
   (d) After the differentiation, wash with PBS and collect the cells in 1 mL Trizol combined with vigorous pipetting to lyse the cells.
   (e) Leave at room temperature for 5 min to disrupt the nucleoprotein complex and subjected to RNA isolation, cDNA conversion, and qPCR analysis following standard manufacturer's protocols.
   (f) Process the other T25 flask postinduction for immunostaining with Insulin and PDX1 expression levels with the standard protocol described earlier.

3.2.3 Cytogenetic Analysis

1. Perform the karyotyping of human placental MSCs at Passages 5 and 25 to verify the chromosomal integrity.
2. Perform metaphase chromosomal preparations using colchicine according to standard procedures at a 400–550 GTG band level.
3. Use an axioplan microscope (Zeiss) to identify and analyze the chromosomes.

4. Analyze the images with a photometric charged coupled device camera and control with a smart capture imaging software.

3.2.4 Immunostaining

1. Incubate the samples for 10 min with PBS containing 0.1–0.25 % Triton X-100 to permeabilize the cells.
2. Wash the cells with PBS.
3. Incubate cells with 1 % BSA in PBST (PBS+ 0.1 % Tween 20)—for 30 min to block unspecific binding.
4. Incubate cells with the primary antibody in 1 % BSA in PBST in a humidified chamber for 1 h at room temperature or overnight at 4 °C.
5. Remove the solution and wash cells in PBS.
6. Incubate cells with the secondary antibody in 1 % BSA for 1 h at room temperature in the dark.
7. Remove the solution and wash with PBS in the dark.
8. Incubate cells on 0.1–1 μg/mL Hoechst for 1 min.
9. Rinse with PBS and observe under the phase contrast microscope using the respective filters.

3.2.5 Total RNA Isolation and cDNA Synthesis

1. The total RNA isolation was performed according to the standard manufacturer's protocol (Trizol—Invitrogen) (*see* **Note 14**).
2. cDNA 1st Strand conversion is performed by following standard manufacturer's protocol (Invitrogen) (*see* **Note 15**). The cDNA was analyzed for expression levels of the respective targets including calibrators (Housekeeping Genes) by a relative qPCR using SYBR chemistry on a 12K Flex Quant Studio (Life Technologies) platform.

3.2.6 Real-Time Polymerase Chain Reaction (QPCR)

1. Calculate the relative expression level by the ddCt method.
2. Normalize Ct values of the targets with that of the Ct values of beta-actin.

3.2.7 In Vitro Tumorigenesis Detection Assay

1. Layer, 0.6 % agar, containing MEM on the surface of 35 mm dish (Corning) and incubate in the laminar hood for 30 min.
2. Mix 2×10^4 MSCs with 0.3 % agar containing MEM and overlay it on the top of 0.6 % agar layer.
3. Incubate the plate in a hood for 20 min.
4. Postincubation, add 500 μL of mesenchymal expansion medium and incubate for 21 days.
5. Top up with 500 μL of fresh media every 7 days. Use HeLa cells as a positive control to evaluate the outcome.

3.2.8 Cell Cycle Analysis

1. Suspend 10^5 placental MSCs in 200 μL of PBS in a 15 mL Falcon tube.
2. Ensure that the cell suspension is mono-dispersed and that cell clumps are absent.
3. Fix the cells by transferring 10 mL of cold methanol drop-by-drop while keeping the tube on ice.
4. Fix the cells for at least 30 min at 4 °C.
5. Centrifuge, discard methanol, and wash with 5 mL PBS.
6. Centrifuge to discard the PBS and resuspend with 50 μg/mL Propidium Iodide with 10 μg/mL RNase A and incubate at 37°C for 5–10 min.
7. Do not wash with PBS. Acquire using a flow cytometer (BD FACS Calibur) and analyze with BD Cell Quest Pro software.

3.2.9 Apoptosis Analysis

1. Carry out the analysis by following the manufacturer's instructions (BD Pharmingen Annexin V) (*see* **Note 16**).
2. Acquire the 7AAD and Annexin V subjected cells in a flow cytometer (BD FACS Calibur) and analyzed with the BD Cell Quest Pro software.

3.2.10 Cell Doubling Time

1. Calculate the population is doubling time from the given formula.

   Population doubling (PD) = $\ln(Nf/Ni)/\ln 2$

   where ln = Natural logarithm; Nf = Final cell count; Ni = Initial cell count
2. Cell population doubling time, Gt = t/PD; t = Time in hours after cell seeding.
3. Obtain the average Ct value by adding the obtained Get values for different experiments divided by some experiments.

3.3 Generation of iPSCs, Characterization, and Differentiation into Neurospheres

1. Nucleofect the plasmids to 10^6 cells with about 3 μg of the plasmid of interest. Follow the standard protocol of the manufacturer (Lonza, Nucleofection) (*see* **Note 17**).
2. Prepare placental feeders freshly on a 60 mm dish 1 day before the nucleofection.

3.3.1 iPSCs Generation

3. Seed 3×10^5 nucleofected placental MSCs onto 35 mm dish containing placental feeders (*see* **Note 18**).
4. Feed the cells with traditional iPSCs Media for the first 2 weeks.
5. On day 15, supplement the iPSCs media with Chir99021, PDO325901, and hLIF until the morphologically typical colonies of iPSCs appear.

3.3.2 Expansion of iPSCs

1. Pick the iPSCs colonies approximately between 20–30 days and expand on matrigel coated dishes using mTeSR media supplemented with Rock inhibitor, Y27632 (10 μM) (*see* **Note 19**).
2. Ensure that the iPSCs are expanded using Dispase.

3.3.3 Differentiation Analysis of iPSCs

Spontaneous Differentiation of iPSCs

1. Transfer the colonies to a non-adherent dish (Corning) and maintain to culture in traditional iPSCs media without βFGF.
2. Culture the embryoid bodies in a suspension culture for 8 days, transfer to gelatin-coated dishes, and culture for another 8 days using same media.
3. Change the media every alternate day.
4. At the end of the 16th day, stain the cells for Nestin (ectoderm), SMA (mesoderm), and AFP (endoderm) expression for differentiation analysis.

Directed Differentiation of iPSCs to Neurospheres

1. Culture day 3 embryoid bodies in Stem Diff: Neural induction media (Stem Cell Technologies) supplemented with 10 μM of rock inhibitor (Y-27632) for 6 days.
2. Change half of the media every day.
3. Transfer the neural aggregate to PLO/Laminin or matrigel coated dishes and culture in neural induction media for another 7–10 days until the appearance of heterogeneous neural structures.
4. Immunostain the neural cells for nestin expression with standard protocols mentioned earlier.

Directed Differentiation of iPSCs to Adipocytes

1. Culture the day 3 embryoid bodies with adipocyte differentiation media (Invitrogen) for 30 days.
2. Change to fresh media every alternate day.
3. At the end of 30 days, stain with oil red O to confirm the fat droplet deposits.

Directed Differentiation of iPSCs to Osteocytes

1. Culture the day 3 embryoid bodies with osteocyte differentiation media (Invitrogen) for 30 days.
2. Replenish with fresh media every alternate day.
3. At the end of 30 days, perform a Von kossa staining procedure to confirm the presence of calcium deposits.

Directed Differentiation of iPSCs into Chondrocytes

1. Culture the day 3 embryoid bodies in a specially fabricated static cell culture system [3].
2. Employ chondrocyte differentiation media (Invitrogen) for 30 days.
3. Add fresh media every 48 h.
4. Confirmed the chondrocyte differentiation by Safranin O or Alcian Blue staining, with the protocols described earlier.

Directed Differentiation of iPSCs into Erythroid Progenitor Cells

1. Perform the Erythroid progenitor differentiation of iPSCs using the protocol as mentioned previously [9].
2. After 30 days, do the erythroid differentiation analysis for the presence of erythroid progenitors by CD71 immunostaining.
3. Perform the Giemsa stain to characterize further the differentiated cells at various stages and other cell types.

Directed Differentiation of iPSCs into Pancreatic Progenitor Cells

1. Employ the suspension cell culture system for pancreatic progenitor differentiation.
2. Culture the day 4 embryoid bodies in P1 media for 6 days.
3. Add Activin A for the first day only and change to fresh media daily.
4. On the 7th day add P2 media to the culture for pancreatic progenitor differentiation and continue for another 6 days.
5. On day 13, add P3 media that is used for the maturation of pancreatic progenitor cells into islets cells.
6. Confirm the presence of pancreatic progenitor cells by confirming the expression of insulin and PDX1 by Immunostaining and dithizone staining.

3.3.4 Immunostaining

As described earlier in Subheading 3.2.4.

3.3.5 Total RNA Isolation and qPCR

As described earlier in Subheading 3.2.5.

3.3.6 Western Blot

1. Lyse the cells with non-denaturating lysis buffer supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Roche).
2. Separate the cell lysates (20 μg) by electrophoresis on 12 % SDS-polyacrylamide gel and transfer to a PVDF (Polyvinylidene Fluoride) membrane (Amersham).
3. Block with and incubate with primary antibody at 4 °C overnight.
4. Rinse the blot with TBST and incubate with horseradish peroxidase (HRP) conjugated secondary antibody at room temperature for 2 h.
5. Detect using ECL prime (Amersham), HRP substrate, and FluroChem E imaging system for detection.
6. Strip the antibodies with stripping buffer if required.

3.3.7 Cytogenetic Analysis

As described earlier in Subheading 3.2.3.

3.3.8 Teratoma Assay

1. Harvest 10^6 cells and mix with Matrigel in a 1:1 ratio.
2. Immediately inject the cells intramuscularly into the hind limb of the SCID mice.
3. Observe the mice once in 3 days for teratoma.

4. Humanely sacrifice the mice after 6–8 weeks depending on the size of the teratoma and carefully excise the same.
5. Fix the excised teratoma with 10 % formalin, embed in paraffin, slice and stain with hematoxylin and eosin.
6. Observe under a light microscope to histologically access the pathology.

3.3.9 Bisulfite Sequencing

1. Resuspend 1 μg of genomic DNA in TE (Tris–EDTA) Buffer and boil at 95 °C for 5 min.
2. Prepare freshly, 3 M NaOH and mix and incubate at 37°C for 20 min for complete denaturation of the DNA.
3. To the above mixture, add freshly prepared sodium bisulfite/hydroquinone solution and mix well.
4. Overlay with mineral oil and subject to incubation for 5 h at 55 °C in the dark.
5. After incubation, carefully transfer sulfonated DNA solution into a fresh tube containing water, 3M Sodium Acetate, and glycogen.
6. Precipitate the sulfonated DNA with isopropanol.
7. Wash the pellet with 70 % ethanol, air-dry and resuspend in an appropriate volume of TE buffer.
8. Desulfonate the DNA with 3 M NaOH and incubate at 37°C for 15 min.
9. Use a mixture of 5 M ammonium acetate and ethanol for precipitation of DNA.
10. Wash the pellet with 70 % ethanol, air-dry, and resuspend in an appropriate volume of TE buffer.
11. Amplify the promoter regions of human OCT3/4 and Nanog by PCR.
12. Subclone the PCR fragments into the pCR 2.1-TOPO vector.
13. Subject five clones of each sample to sequencing using M13 universal primers and analyze for methylation at CpG islands using contig alignment, Vector NTI (Invitrogen).

3.3.10 Microarray Analysis

1. Perform the microarray on an Agilent platform using the human gene expression 8x60K chip.
2. Analyze the images using Gene Spring GX (Agilent). Normalize the data using quantile normalization.
3. Use the R programming algorithm to generate heatmap and hierarchical cluster analysis of significantly up and down regulated genes.
4. Express the values as log2.

5. Use Gene Spring GX and Biointerpreter (Genotypic Technologies) for classifying the genes based on functional categories and pathway analysis.
6. Perform the bioinformatic analysis of protein-protein interactome network and association analysis using STRING 9.1.

3.4 Development of Spinal Cord Injury Mouse Models, Transplantation of Cells, and Evaluation of Recovery

1. Anesthetized adult male/female SCID mice with a combination of ketamine (50 mg/mL) and xylazine (6 mg/mL) by administering it intraperitoneally (*see* **Note 20**).
2. Group the mice as control and transplant.
3. Make a small 1 cm incision and carry out a laminectomy to expose the spinal cord.

3.4.1 SCID Mice Spinal Cord Injury Model

4. Create a spinal crush injury in the mice model applying a force of about 50 KDyne using a pair of forceps (Dumont #5 - Switzerland).
5. For transplant mice group, inject about 1×10^6 cells directly near the injury site into the spinal cord in three regions (3×10^5 cells/site) using disposable Hamilton/BD insulin syringe mounted on an injection device with 3D stabilizer.
6. Inject the cells one at the site of injury, another at the anterior of the injury site, and one posterior to the injury site.
7. Close the incision using anti-bacterial absorbable sutures (Vicryl).
8. Ensure postoperative care is administered daily, monitoring the general health, body weight, food intake, administration of Ringer lactate, analgesic, antibiotics (ciprofloxacin), and expression of bladder and bowel every 12 h.
9. Monitor the mice for 90 days (*see* **Note 21**).

3.4.2 Histology and Immunohistochemistry

1. Harvest the spinal cord carefully from the mice and fix in 10 % formalin.
2. Process the sample using standard protocols and paraffin-embed, followed by longitudinal sectioning.
3. Deparaffinize and stain with hematoxylin-eosin and the respective antibodies (Nestin, GFAP) with the protocols mentioned earlier.
4. Perform the antigen retrieval during immunostaining.

3.4.3 BBB & BMS Score

1. Use the Basso Mouse Scale (BMS) and Basso, Beattie and Bresnahan score (BBB Score) cautiously to evaluate the progress of the mice (*see* **Note 22**).
2. Also, record the limb movements during the assessment.

3.4.4 Transcranial Electrical Stimulation

1. The transcranial electrical stimulator was custom fabricated internally with the assistance of Department of Bioengineering, Christian Medical College.
2. The instrument is used to stimulate the cranium and record the motor-evoked potential from the hind limb muscles.

3.4.5 Bladder Score

1. Access the bladder score that is indicated as the time taken by the mice to regain control of their micturition post-injury.

3.5 In Vivo Tracking of the Transplanted Cells

1. Mix approximately 1×10^6 cells with 0.2 mg/mL ICG in DPBS.
2. Incubate the cells at 37°C for 30 min.
3. Wash the cells twice with DPBS.
4. Resuspend in fresh 100 μL DPBS.
5. Use IVIS imaging station (Perkin Elmer, MA, USA) for in vivo imaging and analysis.

4 Notes

1. Ensure proper sterility and cleanliness of the immediate surroundings. Secure a screen for Blood borne virus (BBV) is done before obtaining consent.
2. Consider all biological samples as potentially hazardous and ensure that proper personal protective equipment (PPE) such as sterile gloves, goggles, apron, cap, etc. is promptly used. The entire sample processing is to be carried out inside a Level 2 Biosafety cabinet.
3. The PBS with Antibiotic is prepared by supplementing PBS (without Ca^+ and Mg^+) with Penicillin (50 Units/mL)/ Streptomycin (50 μg/mL), Gentamycin (5 μg/mL), and Amphotericin B (25 μg/mL). Care should be ensured to employ an optimal dose of antibiotic throughout the isolation protocol to avoid cross-contamination at any step.
4. The maternal and the fetal side of the placenta are to be carefully deciphered. Part the amniotic membrane to expose the cotyledons. Flush liberally with PBS to remove excess blood to reveal the clots, which are to be eliminated.
5. Care should be taken with trypsin digestion, as over-digestion can significantly reduce the cell viability. Prompt mixing of the sample at regular intervals reduces the harsh ill effects of trypsin and enables better penetration and homogenization.
6. RBC lysis should not exceed the recommended total of 10–20 min incubation, and the same is to be carried out at 4 °C or in ice, to ensure minimal stress on the viable cells.

7. Never allow the cells to become over-confluent (above 90 %) as this can cause the cells to attain senescence due to contact inhibition, which is usually irreversible.
8. It would be beneficial to keep remaining cells suspended in cold PBS temporarily in ice to avoid clumping of cells—this can be used as unstained cell control during analysis.
9. The sterility of cells can be compromised during analysis, as they are not for culturing. FACS experiments necessarily require cap-covered sterile FACS tubes (BD, USA) to be used. Another strategy that would come in handy is to use unstained cells post-acquiring for other stains like PI, Hoechst33342, etc. DAPI as a nuclear stain works best post-fixing.
10. Ensure that the confluency of cells do not exceed 60–70 % while seeding for differentiation as the cells can become over-confluent over the entire course of differentiation and can lead to difficulties during analysis/staining/imaging and also can undergo age-related changes due to contact inhibition and even senescence in many cases.
11. Make sure the cells are seeded at an optimal concentration low enough to compensate for the rapid proliferation in 30 days and high enough to ensure communication and to prevent senescence.
12. It is beneficial to screw a filter cap of the T25 flask to the 15 mL Falcon tube to aid in gaseous exchange and at the same time, prevent contamination.
13. Another alternative suggestion would be if an initial volume of 2 mL of media is added and 1 mL of media is removed and replenished by an equal amount to ensure minimal stress to the chondrogenic pellet.
14. Take special precaution when handling RNA due to its high instability due to degradation with RNAses usually found in the air, sweat, and on most surfaces. Standard autoclaving may not destroy the RNAses and would be needed to get rid of regular glass and plasticware by treatment with DEPC water. Add recommended volume of Trizol directly after aspirating media to minimize degradation by RNases. Ensure sufficient volume of Trizol to prevent contamination by DNA during phase separation. DEPC-treated/Nuclease-free water is recommended.
15. It is to be made sure that a DNase treatment is performed on the RNA before a cDNA conversion or that the primers span an exon-exon junction to prevent contaminating DNA during RNA isolation from interfering with the expression analysis.
16. Care should be taken to be as gentle as possible when preparing a single cell suspension as even the slightest damage to membrane architecture can expose the inner Phosphatidyl Serine to the surface giving false-positive results.

17. The plasmids to be used are strict to be endotoxin-free, as this can significantly affect the reprogramming phenomenon. This is achieved by subjecting them to endotoxin eliminator/resin or isolate them using standard endo-free plasmid isolation kits.
18. To prepare the feeders treat the cells with mitomycin C (10 μg/mL) and incubate for 3 h at 37°C. After incubation aspirate, the mitomycin C solution, wash with PBS twice, and trypsinize the cells. Seed the 2×10^5 cells onto 1 % gelatin coated 35 mm dish.
19. Avoid picking the partially reprogrammed colonies for expansion. One can use StainAlive Tra 181 (DyLight 488) to facilitate the selection of properly reprogrammed colonies.
20. The anesthesia concentration has to be accurate as an overdose will considerably affect the animal and may also lead to death. The concentration we have optimized is four units on an insulin syringe per 10 g body weight. Ensure that the mouse is completely lacking any pinch reflexes before starting the procedure.
21. Many parameters are to be continuously monitored during the post-injury period. Serum levels of creatinine and urea indicative of kidney function, general activity including irritability, CRP—indicative of inflammation, liver transaminases, motor function, and a normal body weight monitor are to be accessed over the entire course of the experiment.
22. The BMS/BBB score is to be accessed routinely by a group of blinded and nonblinded evaluator. This is to rule out all instances and probability of any bias when determining the BMS/BBB score.

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Chapter 9

Therapeutic Application of Human Wharton Jelly Mesenchymal Stem Cells in Skin Injury of SCID

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Abstract

Mesenchymal stem cells (MSCs) are blossoming as a credible source for regenerative medical applications. The use of fetal MSCs is gaining momentum for therapeutic use. The ease of isolation, enhanced characteristics, and immunomodulation properties renders the utilization of fetal MSCs for numerous clinical applications. In this article, we will demonstrate a step-by-step protocol for isolation of Wharton's jelly MSCs (WJMSCs) from the human umbilical cord matrix, preparation of human platelet lysate, fabricating amniotic membrane scaffold and mice model to study skin regeneration using a combination of MSCs and decellularized amniotic membrane scaffold.

Key words Mesenchymal stem cells, Platelet lysate, Amniotic membrane, Scaffold, Skin injury, Indocyanine green

1 Introduction

Skin injury is one of the most common forms of injury. Skin injury might occur as acute and chronic injury. Wound healing is a convoluted process comprising of a cascade of molecular and cellular events involving multiple cell types, soluble factors, and matrices [1]. Emerging studies have indicated that MSCs could augment repair of the injured skin [2]. In our study, we have effectively optimized the use of bioengineered amniotic membrane scaffold for tissue engineering [3].

MSCs are a class of a particular set of stem cells that exhibit self-renewal and multipotency. They can be isolated from both adult and fetal tissues. The multipotency, anti-inflammatory, and immunomodulatory properties render the MSCs as the ideal choice for cell therapy applications [3, 4]. Invasive procedure and low expansion capability are the stumbling roadblocks in using adult MSCs. The divestiture of adults MSCs can be overcome by the use of fetal MSCs. The attributes such as ease of isolation, rapid expansion, and high passage numbers rank fetal MSCs in higher echelon compared

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with adult MSCs [3, 4]. We have standardized the protocol for isolation of Wharton jelly MSCs (WJMSCs) [3].

Fetal bovine serum (FBS) is routinely used in cell culture applications. However, the xenogeneic substances present in the FBS might contaminate the culture. Human platelet lysate (HPL) is emerging as an effective replacement to fetal bovine serum. The HPL are rich in human growth factors that are suitable for dynamic cell culture [3, 5]. Although previous studies discovered batch-to-batch variation in HPL, the proliferation of the MSCs in HPL was superior compared to FBS [5]. For culturing of WJMSCs, we have used pooled platelet lysate.

Growing evidence suggests that MSCs seeded onto artificial dermal scaffolds could help in augmenting the tissue regeneration process [6]. The data from our study indicate that use of decellularized amniotic membrane scaffold in combination with MSCs augmented the regeneration of the skin when compared with the use of MSCs alone.

This protocol describes all the necessary steps to be followed to study the regeneration of skin using mice models.

2 Materials

Unless mentioned all the chemicals and reagents were procured from Sigma.

2.1 Animals

1. Black SCID mice (B6.CB17-prkdcScid/SzJ) can be used for the skin injury model.
2. All mice kept separately in cages and routine animal care performed in animal house.

2.2 Anesthesia System

1. Ketamine (50 mg/mL).
2. Xylazine (6 mg/mL).
3. Isoflurane.
4. O_2 cylinders.

2.3 Derivation of Wharton's Jelly MSCs

1. Ethylene gas sterilized tissue collection bag.
2. Sterilized tissue processing container/tray.
3. Straight surgical sharp scissors.
4. Scalpel holder and surgical scalpel.
5. Sterile metal sieve (250 μm).
6. Phosphate buffered saline (PBS) (pH 7.4, Without Mg^{2+} and Ca^{2+}): 137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 2 mM KH_2PO_4.
7. Dulbecco's phosphate buffered saline (DPBS) without Ca^{2+} and Mg^{2+}.

8. Penicillin (100 U/mL), Streptomycin (100 μg/mL) and Amphotericin B (2.5 μg/mL).
9. 12.5 U/mL Collagenase 1 (in α-MEM with 1 % antibiotics and without FBS).
10. Dispase (2 mg/mL).
11. Red Blood Cell (RBC) lysis buffer: (0.1 mM EDTA, 155 mM NH_4Cl and 12 mM $NaHCO_3$).
12. Ice cubes.
13. Mesenchymal expansion medium: Alpha Minimal Essential Medium (α-MEM), 5 % Human platelet lysate (HPL) L-glutamine (1 mM) and Penstrep (1 %).
14. Trypsin (0.05 %).
15. Freezing Media: complete α-MEM with 5 % DMSO.
16. Isopropanol bath.
17. Liquid nitrogen.

2.4 In Vitro Differentiation of Wharton's Jelly MSCs

1. Adipocyte differentiation media (Invitrogen, CA, USA).
2. Oil Red O stain.
3. 4 % paraformaldehyde.
4. Sterile water.
5. Isopropanol (60 %).
6. Osteocyte Differentiation media (Invitrogen, CA, USA).
7. Vonkossa stain.
8. Precooled methanol.
9. Silver Nitrate (5 %).
10. UV light.
11. Sodium thiosulphate (5 %).
12. Chondrocyte differentiation media (Invitrogen, CA, USA).
13. Insulin syringe.
14. 10 % formalin.
15. Merchrome staining.
16. Paraffin.
17. Safranin O.
18. Alcian blue.
19. Xylene.
20. DPX mounting media.
21. Neuronal induction medium (Serum-free α-MEM and 5 mM β-mercaptoethanol).
22. Retinal differentiation medium (Mesenchymal expansion media, 50 μm Taurine and 1 mM β-mercaptoethanol).

23. Gelatin.
24. Pancreatic β cell differentiation media (10 mM nicotinamide and 1 mM β-mercaptoethanol).

2.5 In Vitro Characterization of Human Wharton's Jelly MSCs for the Surface Marker Expression

1. Antibodies (eBioscience Inc.): anti-human CD14 FITC, anti-human CD34 FITC, anti-human CD45 FITC, anti-human CD29 FITC, anti-human CD73 FITC, anti-human CD90 PE, antihuman CD105 APC, anti-human HLA-DR FITC, Mouse IgG1 K FITC isotype control Mouse IgG1 K PE isotype control and mouse IgG1 K APC isotype control.
2. Cell Quest software (Becton Dickinson, USA).

2.6 Secondary Characterization of Human Wharton's Jelly MSCs

1. Matrigel (BD).
2. Agar (0.6 %).
3. Agar (0.3 %).
4. HeLa cells for positive control (Tumorigenesis detection assay).
5. Ice cold Methanol.
6. RNaseA (10 μg/mL).
7. Propidium iodide (50 μg/mL).
8. Apoptosis detection kit (BD pharmingen).
9. Tissue culture flask (25 cm^2).
10. 2′,7′-Dichloro Fluorescin Diacetate (DCFDA) (10 μM).
11. Hydrogen peroxide (H_2O_2) (50 μM).
12. Colchicine (0.05 %).

2.7 Immunostaining

1. BSA (0.1 %).
2. Paraformaldehyde (4 %).
3. Triton X 100 (0.2 %).
4. Blocking buffer (PBS and 0.1 % BSA).
5. Hoechst 33342.
6. Antibodies: NG2 Alexa Fluor 488 (eBioscience), SMA (abcam), Rhodopsin (abcam), Insulin (Cell Signaling), and PDX1 Alexa Fluor 647 (eBioscience).

2.8 Total RNA Isolation, cDNA Synthesis and Real-Time Quantitative PCR

1. Trizol reagent (Invitrogen, CA, USA).
2. Superscript III first-strand synthesis system (Invitrogen, CA, USA).
3. F-410 DyNAmo HS SYBR Green master mix (Thermo Scientific, MA, USA).
4. Primers (Table 1).

Table 1
Oligo sequences used for quantification of endogenous gene expression by qRT PCR

Primers	Sequence 5′–3′
Beta Actin Fwd	CCT TCC TGG GCA TGG AGT CCT
Beta Actin Rev	GGA GCA ATG ATC TTG ATC TTC
Human IDO Fwd	CAAAGGTCATGGAGATGTCC
Human IDO Rev	CCACCAATAGAGAGACCAGG
Human PGE2 Fwd	GAC CGC TTA CCT GCA GCT GTA C
Human PGE2 Rev	TGA AGT TGC AGG CGA GCA
Human TGFβ1 Fwd	CCCAGCATCTGCAAAGCTC
Human TGFβ1 Rev	GTCAATGTACAGCTGCCGCA
Human TSG6 Fwd	GGCCATCTCGCAACTTACA
Human TSG6 Rev	CAGCACAGACATGAAATCCAA

2.9 Immunological Characterization

1. IL-1β (10 ng/mL).
2. TNFα (10 ng/mL).
3. INFα-2b (150 U/mL).

2.10 Human Platelet Lysate Preparation

1. Platelet Units (Hospital blood bank).
2. 2 U/mL Heparin.
3. 0.2 μm PVDF membrane filter (Millipore, Billerica, MA, USA).

2.11 Amniotic Membrane Isolation and Decellularization

1. Amniotic membrane.
2. 1× PBS.
3. Tris buffer (10 mM).
4. EDTA (0.1 %).
5. Aprotonin (10 kiu/mL).
6. SDS (0.03 %).
7. Tris–HCl (50 mM).
8. DNase 1 (50 U/mL).
9. RNase (1 U/mL).
10. Magnesium chloride (10 mM).
11. Bovine serum albumin (50 μg/mL).
12. Peracetic acid (0.1 %).
13. Ethanol (70 %).

14. Penicillin/streptomycin (1 %).
15. Amphotericin B (2.5 μg/mL).

2.12 Mice Skin Injury and Transplantation

1. Heating pad.
2. Shaving razor.
3. Hair removal cream.
4. Sterile sharp surgical scissors.
5. Sterile forceps.
6. Syringe.
7. Human WJMSCs (1×10^6).
8. Amniotic membrane scaffold seeded with human WJMSCs (1×10^6).
9. Suture (20 mm Vicryl, ETHICON VP2437).
10. 2 mg/mL ciprofloxacin injection (liplox).
11. Analgesic: meloxicam (5 mg/mL), paracetamol (150 mg/mL).

2.13 In Vivo Tracking of Labeled Human WJMSCs

1. ICG (0.2 mg/mL).
2. Living Image Software 4.0 (PerkinElmer, MA, USA).

2.14 Consumables

1. Centrifuge tubes 15 mL and 50 mL.
2. Tissue culture flasks: T25 cm^2, T75 cm^2 and T150 cm^2 flask.
3. FACS tubes (Becton Dickinson, NJ, USA).
4. Cell culture plates: 24 well, 96 well.
5. Cell culture dishes: Cell culture treated dishes (100 mm, 60 mm and 35 mm), ultra low attachment dish (35 mm).
6. Serological pipette (5 mL, 10 mL and 25 mL).
7. Pipette filter guarded tips (10 μL, 200 μL and 1000 μL).

2.15 Instruments

1. Centrifuge.
2. Biosafety cabinet 2.
3. Inverted microscope (Leica, Wetzlar, Germany).
4. CO_2 incubator.
5. FACS Calibur instrument (Becton Dickinson, NJ, USA).
6. Quant Studio 12K flex real-time PCR system (Invitrogen, CA, USA).
7. −80 °C freezer.
8. Liquid nitrogen storage container.
9. Water bath (37 °C).

10. Vortex instrument.
11. Zeiss axioplan microscope equipped with photometric charged coupled device camera.
12. Pipette gun.
13. Neubauer Haemocytometer.
14. Fluorescence microscope (Leica DMI 6000B) equipped with Digital camera (DFC360FX) (Leica, Wetzlar, Germany).
15. Leica imaging software.
16. Pipette gun.
17. Pipette (1–10 μL, 20–200 μL and 1000 μL).
18. IVIS imaging station (Perkin Elmer).

3 Methods

3.1 Collection and Isolation of Human Wharton Jelly MSCs

1. Ethical approval for the study was obtained from the Institutional Review Board (IRB), Christian Medical College, Vellore (*see* **Note 1**).
2. Consent from the patients undergoing full-term elective caesarean was obtained for collection of human umbilical cord sample (*see* **Note 2**).
3. Collect umbilical cord sample in ethylene gas sterilized tissue collection bag and keep on ice during transport to the laboratory for the processing (Fig. 1a) (*see* **Note 3**).
4. Transfer the full-length umbilical cord into the autoclaved tissue-processing container containing 1× PBS (Fig. 1b) (*see* **Note 3**).
5. Wash the umbilical cord with 1× PBS containing antibiotics (penicillin and streptomycin) and antifungal solution (Amphotericin B) for devoid of contamination before processing the tissue for isolation of cells (Fig. 1c,d) (*see* **Note 4**).
6. Cut the umbilical cord into pieces of 5 cm (Fig. 1e) (*see* **Note 5**).
7. Wash the umbilical cord tissue pieces with Dulbecco's phosphate buffer saline (DPBS) (Fig. 1f).
8. Remove the arteries and vein in the umbilical cord by cutting along the horizontal axis [7].
9. Mince the umbilical cord tissue sample mechanically (Fig.1g,h) (*see* **Note 6**).
10. Wash the minced tissues twice with DPBS (Fig. 1i,j).
11. Transfer the minced tissue into sterile 50 mL centrifuge tube containing collagenase 1 and incubate in shaking water bath at 37 °C for 12 h (Fig. 1k,l).

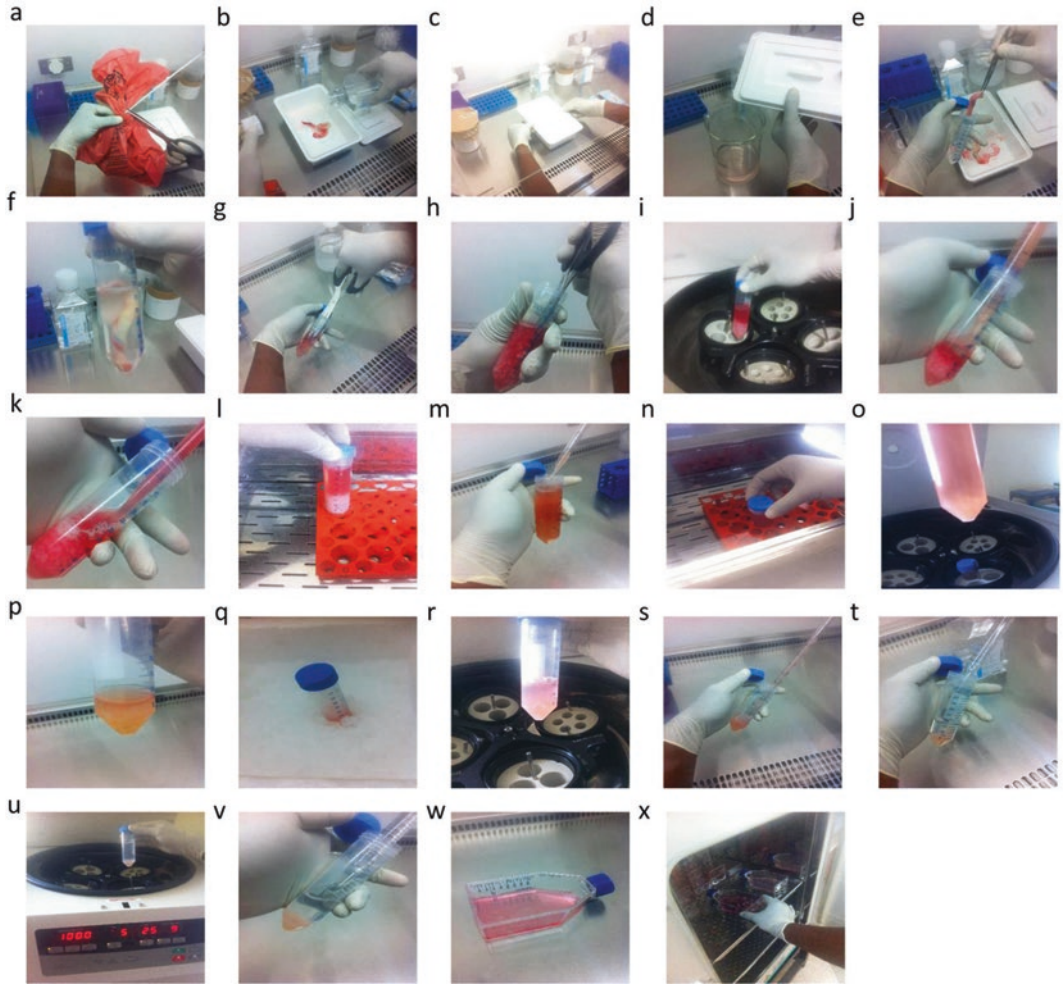

Fig. 1 Overview of isolation of Wharton's jelly MSCs. (**a**) Human umbilical cord tissue collected and transferred to lab in sterile tissue collection bag. (**b**) Tissue transfered from bag to sterile tissue processing container. (**c**-**d**) Tissue washed with PBS. (**e**) Tissues cut into pieces and then transfered into centrifuge tubes. (**f**) Tissue pieces washed with DPBS. (**g**-**h**) Tissue pieces minced mechanically with sterile surgical scissor. (**i**) Minced tissues collected by centrifugication. (**j**) Supernatant removed from the centrifuge tube leaving the pellet intact. (**k**) MEM containing collagenase 1 enzyme added to the pellet. (**l**) Centrifuge tube incubated in shaking waterbath. (**m**) Dispase enzyme added to the centrifuge tube. (**n**) Centrifuge tube incubated in shaking waterbath. (**o**) Enzymatically digested tissue collected by centrifugation. (2 **p**) Supernatant removed from the centrifuge tube and RBC lysis buffer added to the pellet. **q**) Centrifuge tube incubated in ice. (**r**) Tissues collected by centrifugation. (**s**) Supernatant removed from the centrifuge tubes. (**t**) Antibiotics containing DPBS added to the pellet. (**u**) Tissues collected by centrifugation. (**v**) Supernatant removed from the centrifuge tube. (**w**) Pellet seeded into tissue culture flask. (**x**) Tissue culture flask incubated in CO_2 incubator

12. After the incubation add dispase (2 mg/mL) into the same tube and then incubate the sample for 2 h at 37 °C in shaking water bath (Fig. 1m,n) (*see* **Note 7**).
13. Pass the enzyme digested umbilical cord tissue sample into the 250 μm metal sieve (*see* **Note 8**).

14. Transfer the metal sieve retained tissue sample into the 50 mL centrifuge tube with sterile forceps.
15. Add 25 mL of DPBS into the tube containing tissue sample and mix properly.
16. Centrifuge the tube for 10 min at 300 × *g*/4 °C (Fig. 1o).
17. Remove the supernatant and add 10 mL of RBC lysis buffer into the tube (Fig. 1p).
18. Mix properly and incubate the tube on ice for 15 min with intermittent mixing at the time interval of 3 min (Fig. 1q).
19. Centrifuge the tube for 10 min at 300 × *g*/4 °C (Fig. 1r).
20. Remove the supernatant and add 25 mL of DBPS containing 1 % penicillin/streptomycin and 2.5 μg/mL of amphotericin B (Fig. 1s,t) (*see* **Note 9**).
21. Centrifuge the tube for 10 min at 300 × *g*/4 °C (Fig. 1u).
22. Remove the supernatant and transfer the tissue sample into T150 cm^2 flask added 30 mL of mesenchymal expansion medium (Fig. 1v,w).
23. Transfer the flask into humidified (37 °C and 5 % CO_2) incubator (Fig. 1x).
24. Replace one third of the old media on every 5th day with fresh media.
25. Colonies of mesenchymal stromal cells will appear in the flask by 7–14 days.
26. For passaging trypsinize the cells and split it into 1:2 ratio. Add fresh MSC maintenance media once in 3 days (*see* **Notes 10** and **11**).

3.2 Characterization of Human Wharton Jelly MSCs

3.2.1 Flow Cytometer-Based Surface Marker Analysis

1. Once mesenchymal stromal cells in culture flask reach 70–80 % confluency, wash the cells twice with 10 mL of 1× PBS.
2. Add 1 mL of 0.05 % trypsin-EDTA into the flask and make sure that it covers the entire surface of the flask.
3. Incubate the flask in a 37 °C incubator for 5 min.
4. After the incubation observe the cells under phase contrast microscope for the dissociation of monolayer cells into single rounded cells.
5. Add 10 mL of α-MEM to inactivate the trypsin activity and then transfer the suspension of cells into 15 mL centrifuge tube.
6. Centrifuge at 160 × *g* for 5 min to pellet down the cells.
7. Remove the supernatant and resuspend the cells in 1× PBS.
8. Transfer approximately 1 × 10^5 cells/tube into FACS tubes.
9. Add 5 μL of appropriate antibodies conjugated with specific fluorophores to the cells and incubate in the dark at room temperature for 20 min.

10. Use unstained and appropriate IgG isotype antibodies as a control.
11. After incubation wash the cells with 1× PBS to remove the excess unbound antibodies.
12. Resuspend the cells in 1× PBS and acquire by FACS Calibur instrument.
13. Record minimum 10^4 events for the analysis of surface marker expression by flow cytometer.
14. Analyze the results using Cell Quest software.

3.2.2 Differentiation Analysis (See Note 12)

1. Adipocyte differentiation
   (a) Use commercially available adipogenic induction medium for adipocyte differentiation of human WJMSCs.
   (b) One day before the differentiation seed about 5×10^4 cells/well into a 24-well plate containing mesenchymal expansion media.
   (c) Remove the old media and add adipogenic induction medium to the cells and incubate the plate at 37 °C in a humidified 5 % CO_2 incubator for 30 days.
   (d) Every second day change the adipogenic induction medium.
   (e) Visualize oil droplets (an indicator of adipogenic differentiation) by Oil Red O staining.
   (f) Fix the cells with 4 % paraformaldehyde and wash the fixed cells with sterile water.
   (g) Incubate the cells with 60 % isopropanol at room temperature.
   (h) Add 0.5 % Oil Red O in isopropanol into the cells and incubate at room temperature for 20 min.
   (i) Sequentially wash the stained cells with 60 % isopropanol and sterile water.
   (j) Visualize under a microscope and image the stained cells for the accumulation of lipid droplets.
2. Osteocyte Differentiation
   (a) Use osteoblast differentiation medium for osteocyte differentiation of human WJMSCs.
   (b) Seed 5×10^4 cells/well into a 24-well tissue culture plate containing mesenchymal expansion media.
   (c) Add osteoblast differentiation media into the cells and incubate them at 37 °C in a humidified 5 % CO_2 incubator for 30 days.
   (d) Change the osteoblast induction medium every second day.

(e) Confirm the presence of extracellular calcium deposits by Vonkossa staining.

(f) Fix the cells in precooled methanol.

(g) After fixing wash the cells with DPBS.

(h) Treat the cells with 5 % silver nitrate solution in water and expose the plate to UV light under the laminar hood for 1 h.

(i) Wash the stained cells with sterile water and incubate with 5 % sodium thiosulphate in water at room temperature for 2 min.

(j) Rinse the sample with sterile water.

(k) Observe the cells under microscope and image the extracellular calcium deposition.

3. Chondrocyte differentiation

(a) Use chondrogenic differentiation medium for chondrocyte differentiation of human WJMSCs.

(b) Centrifuge about 1×10^6 cells in a 15 mL Falcon centrifuge tube at $650 \times g$ to obtain the micropellet of cells.

(c) Add chondrogenic differentiation medium through the side of the tube using insulin syringe without disturbing the pellet and incubate at 37 °C in a humidified 5 % CO_2 incubator for 30 days.

(d) Replace the closed cap of the centrifuge tube with filtered cap of the T25 cm^2 flask to allow an adequate gas exchange.

(e) Carefully change the chondrocyte differentiation media every second day without disturbing the pellet.

(f) Confirm the chondrocyte differentiation by staining the sections of the pellet by safranin O and 3 % alcian blue.

(g) Fix the micropellet cells in 10 % formalin and stain with merchrome and subsequently, embed in paraffin.

(h) Stain the deparaffinized 5 μm sections with 0.1 % safranin O or 3 % alcian blue.

(i) Rinse the sections after staining with distilled water.

(j) Air-dry the sections at room temperature and immerse in xylene.

(k) Mount sections using DPX mounting media and observe under the microscope.

4. Tubular assay

(a) Thaw the Matrigel at 4 °C overnight.

(b) Aliquot 50 μL/well of matrigel into 96-well plate using precooled tips.

(c) Centrifuge the plate for 5 min at 300 × g.

(d) Incubate at 37 °C for 30 min to allow matrigel to polymerize.

(e) Seed 1 × 105/well human WJMSCs into the matrigel-coated 96-well plates containing mesenchymal expansion medium.

(f) Incubate the cells for 6 h under hypoxia condition at 37 °C.

(g) Observe tubular structures under the microscope.

5. Neural

(a) Use neuronal induction media to differentiate the human WJMSCs into neuron [8].

(b) Seed 5 × 10^5 cells and add serum free α-MEM containing 5 mM of β mercaptoethanol.

(c) Culture the cells for 6–9 h at 37 °C in humidified 5 % CO_2 incubator.

(d) Fix the cells and confirm the neural differentiation by immunostaining for Neuroglia2 (NG2) marker expression.

6. Retinal

(a) Use retinal differentiation media (α-MEM supplemented with 50 μM of taurine and 1 mM of β mercaptoethanol) for differentiation of human WJMSCs into retinal progenitor cells.

(b) Seed 1 × 10^5 cells and add retinal differentiation media.

(c) Incubate at 37 °C in a humidified 5 % CO_2 incubator for 8 days.

(d) Change the media on every second day.

(e) Confirm the retinal progenitor differentiation by immunostaining for rhodopsin marker expression.

7. Pancreatic progenitor cell differentiation

(a) Treat the T25 cm^2 culture flask with gelatin.

(b) Seed 5 × 10^5 cells into the gelatin-coated flask-containing complete α-MEM supplemented with 10 mM nicotinamide and 1 mM of β mercaptoethanol.

(c) Incubate at 37 °C in a humidified 5 % CO_2 incubator for 24 h.

(d) After the preinduction, treat the cells with α-MEM (without FBS) supplemented with 10 mM nicotinamide and 1 mM of β mercaptoethanol for 6 h.

(e) Subsequently, add induction media with FBS for 18 h.

(f) Fix the cells and confirm the pancreatic progenitor cell differentiation by immunostaining for the expression of insulin and PDX1 markers.

3.2.3 Cytogenetic Analysis

1. To check the integrity of chromosomes in human WJMSCs perform karyotyping cytogenetic analysis.
2. Perform meta chromosomal preparation with colchicine using standard procedure at 400–500 GTG band level.
3. Use a microscope to analyze the chromosome integrity.

3.2.4 Immunostaining

1. Block the cells cultured in 6-well plates with PBS (without Ca^{2+} and Mg^{2+}) containing 0.1 % BSA.
2. Fix the cells with 4 % paraformaldehyde and then treat cells with 0.2 % Triton X-100 for permeabilization.
3. Incubate the cells with appropriate primary antibody.
4. Block with PBS supplemented with 0.1 % BSA.
5. Incubate the sample with the fluorescent dye-conjugated secondary antibody.
6. Counterstain with nuclear stain Hoechst 33342.
7. Image the sample under the fluorescence microscope and analyze the results with appropriate imaging software.

3.2.5 Total RNA Isolation and cDNA Synthesis

1. Isolate the total RNA from the sample using the Trizol reagent according to the manufacturer's protocol.
2. Prepare the cDNA from the isolated RNA by Superscript III first strand synthesis system according to the manufacturer's protocol.

3.2.6 Real-Time Polymerase Chain Reaction (qPCR)

1. Use SYBR green master mix for the real-time PCR analysis in Quant Studio 12K flex real-time PCR system for Real-time quantitative PCR according to the manufacturer's protocol.
2. Use beta-actin expression for normalizing the gene expression levels.

3.2.7 In-Vitro Tumorigenesis Detection Assay

1. Add 0.6 % agar containing MEM on the surface of 35 mm dish as a layer and incubate in the laminar hood for 30 min.
2. Mix 2×10^4 human WJMSCs into the 0.3 % agar containing MEM and overlay on the top of 0.6 % agar layer.
3. Incubate the dish for 20 min in a laminar hood.
4. Add 500 μL of mesenchymal expansion media and incubate at 37 °C in a humidified 5 % CO_2 incubator for 21 days.
5. Add 500 μL of fresh mesenchymal expansion media every 7 days.
6. Use HeLa cells as a positive control for in vitro tumorigenesis detection assay.

3.2.8 Cell Cycle Analysis

1. Fix 1×10^6 human WJMSCs with ice-cold methanol by adding drop by drop with proper intermittent vortexing.
2. Incubate at 4 °C for 2 h.

3. Centrifuge at 300 × *g* for 10 min.
4. Remove the methanol and wash twice with 1× PBS.
5. Resuspend the cells in 100 µL of 1× PBS and treat the cells with RNaseA (10 µg/mL) and stain with propidium iodide (50 µg/mL).
6. Incubate in ice for 5–10 min.
7. Analyze the sample by flow cytometer.

3.2.9 Apoptosis Analysis

Use apoptosis detecting Annexin V kit (BD Pharmingen) for apoptosis analysis of human WJMSCs according to the manufacturer's protocol.

3.2.10 Cell Doubling Time

Population doubling time was calculated using the formula.

$$\text{Population doubling}(PD) = \ln(Cf / Ci) / \ln 2$$

where ln = Natural logarithm; Cf = Final cell count; Ci = Initial cell count

1. Cell population doubling time, Nt = t/PD; t = Time in hours after cell seeding.
2. Obtain the average Nt value by adding the obtained Nt values for different experiments divided by some experiments.

3.2.11 Redox Potential Analysis

1. Seed about 1 × 10^5 human WJMSCs into the T25 cm^2 flask.
2. Trypsinize the cells with 0.05 % trypsin-EDTA.
3. Wash the pellet with DPBS.
4. Add media containing 10 µM DCFDA into the suspension of cells and incubate at 37 °C for 30 min.
5. Add 50 µM of H_2O_2 along with 10 µM DCFDA into another cell suspension as a positive control for redox potential.
6. Analyze the cells by flow cytometry.

3.2.12 Immunological Characterization

1. Seed 1 × 10^5 cells/well into 6-well plates.
2. Add proinflammatory cytokines, L-1β (10 ng/mL), TNFα (10 ng/mL) and INFα-2b (150 U/mL) directly in the media.
3. Incubate for 48 h.
4. Trypsinize and store the cell pellet in trizol.
5. Estimate the relative gene expression of immunomodulatory factors TGFβ1, IDO, TSG6 and PGE2 using (qPCR).

3.3 Preparation of Human Platelet Lysate

1. Pool human platelet obtained from hospital blood bank and distribute them into 50 mL centrifuge as 45 mL aliquots (*see* **Note 13**) [9].
2. Freeze the aliquots at −80 °C freezer overnight.

3. Thaw human platelet aliquots by incubating them in 37 °C water bath for 10 min.
4. Repeat freezing and thawing cycles twice for platelet lysis to occur.
5. Add 2 U/mL of heparin to the lysate to avoid gelatinization process.
6. Remove membrane fragments from the platelet lysate by centrifuging at 2600 × *g* for 30 min.
7. Pass platelet lysate through 0.2 μm PVDF membrane filters and aliquot into the 50 mL centrifuge tube.
8. Store aliquot at −80 °C freezer (*see* **Note 14**).

3.4 Fabrication of Amniotic Membrane Matrix

1. Cut open amniotic membrane from placenta with sterile scissors and transfer into 100 mm culture dish.
2. Wash the amniotic membrane with 1× PBS before the decellularization process.
3. Incubate membrane in hypotonic 10 mM Tris buffers supplemented with 0.1 % EDTA and 10 kiu/mL aprotinin overnight [10].
4. Treat the tissue in solution containing 0.03 % SDS, 0.1 % EDTA and 10 kiu/mL aprotinin for 24 h.
5. Wash membrane with 1× PBS.
6. Immerse-treated membrane in reaction buffer supplemented with 50 mM Tris–HCl, 50 U/mL DNase 1, 1 U/mL RNase, 10 mM magnesium chloride and 50 μg/mL bovine serum albumin for 3 h.
7. Sterilize decellularized amniotic membrane scaffold by sequentially washing with 0.1 % peracetic acid, 70 % ethanol and 1× PBS containing 1 % Penicillin/Streptomycin and 2.5 μg/mL of Amphotericin B (*see* **Note 15**).

3.5 Generation of Mouse Skin Injury Model and Transplantation of Human Wharton Jelly MSCs

1. Anesthetize black SCID mice (B6.CB17-prkdcscid/SzJ) by administering 50 mg/mL ketamine—6 mg/mL xylazine mixture intra-peritoneally (*see* **Notes 16** and **17**).
2. Remove the hair from the skin of the mice by shaving razor and make the area smooth by applying hair removal cream.
3. Cut open the dorsal skin about 1 cm^2 with sterile scissors to create full skin excision wound.
4. In one group of mice ($n = 3$) inject 1×10^6 human WJMSCs resuspended in 100 μL of 1× PBS into all the four directions on the skin injury.
5. Seed 1×10^6 human WJMSCs onto the amniotic membrane scaffold (24–48 h prior) and suture the fabricated scaffold onto the surface of the damaged skin of the mice ($n = 3$).

6. Mice with skin injury treated neither with cells nor amniotic membrane scaffold grafting serve as control group.
7. To rule out the variabilities repeat the experiment at least thrice (*see* **Note 18**).
8. Subject the wound area of all the group mice to histopathology 14 days after cell transplantation or amniotic membrane scaffold grafting.

3.6 In Vivo Tracking of Human Wharton Jelly MSCs

1. Add 0.2 mg/mL final concentration of ICG into the 1× PBS suspension having 1 × 10^6 human WJMSCs and mix properly (*see* **Note 19**) [11].
2. Incubate at 37 °C for 30 min for appropriate labeling of cells.
3. Wash twice with DPBS and resuspend the cells in 100 μL of DPBS.
4. Inject the ICG labeled cells into the skin wound.
5. Anesthetize animals using isoflurane anesthesia system.
6. Track the fate of transplanted ICG labeled Wharton Jelly MSCs in mice by in vivo near infrared fluorescence imaging in IVIS system (*see* **Note 20**).

4 Notes

1. It is mandatory to obtain necessary regulatory approval before using human tissues for the experiment.
2. Collect full-term placenta only from patients undergoing elective caesarean. Placenta from normal delivery is not very sterile. Get the patients consent 1–2 days before surgery.
3. Use two sterilized bags. Place the placenta in one bag and fasten it with cable tag before placing and securing it in the second bag. The placenta-containing bag can be placed safely at 4 °C for up to 3–4 h.
4. It is mandatory to wash the tissue sample with an antibacterial and antifungal solution before processing. Wash the tissue sample up to three times.
5. Take only 5 cm cord sample per 50 mL tube as enormous tissue size may reduce the enzymatic activity.
6. Inappropriate mincing may minimize the enzymatic activity.
7. Add dispase 2 mg/mL directly into the collagenase digested tissue sample. It is not necessary to remove collagenase before treating the tissue samples with dispase. Do not use trypsin instead of dispase as it lowers the derivation of the MSCs from the cord tissue sample.

8. This step is not essential. Proceed directly with centrifugation **step 14**.
9. Treat the tissue with an anti-fungal and antibacterial solution if the sample is not washed with an anti-fungal and antibacterial solution at **step 5**.
10. Excess cells could be subjected to freezing with the help of freezing media and place the cryovials at −80 °C overnight in isopropanol bath before storing the sample in liquid nitrogen.
11. Overgrowth of the cells might lead to differentiation and senescence of the cells.
12. According to the ISCT guidelines adipocyte, osteocyte and chondrocyte differentiation is enough to exhibit the multipotency differentiation potential of MSCs. However, we have carried out translineage differentiation of MSCs to test the plasticity of MSCs.
13. The plasma separated from the blood expires within 72 h for clinical application. Hence, expired blood plasma (<3 months old) can be pooled to prepare platelet lysate.
14. The frozen human platelet lysate can be used up to 6 months.
15. The sterilized amniotic membrane can be cut into smaller pieces and used immediately or can be stored at 4 °C in PBS/−80 °C in freezing media until use.
16. We have used ketamine and xylazine for anesthetizing the animals during surgery. Alternately isoflurane-based anesthesia system could be used with 2–3 % isoflurane and 4 % oxygen.
17. We have to use SCID mice to avoid immune rejection of the transplanted human cells. Any immunocompromised mice are acceptable for the experiment.
18. Postoperative care of the mice is carried out by administering the animals with ringer lactate, analgesic (meloxicam and paracetamol), and antibiotics (ciprofloxacin) for 7 days postsurgery.
19. Increased concentration of ICG leads to cytotoxicity of the cells.
20. The cells can be tracked real time by quantified using emission of photons using living image software (PerkinElmer).

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Chapter 10

Isolation, Characterization, and Expansion of Cancer Stem Cells

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Abstract

The ability to isolate, characterize, and expand distinct tumor cell populations from primary tissue or xenografts is vital to identifying molecular mechanisms specific to cancer stem cells. Once cells have been extracted from tissue, there are multiple methods by which they can be sorted and cultured. We will describe the approaches that can be taken from cancer stem cell isolation through expansion, including Magnetic-activated Cell Sorting (MACS), Fluorescence-activated Cell Sorting (FACS), the use of reporter systems, and various cell culture methods.

Key words Cancer stem cell, MACS, FACS, Sorting, Reporter system, Xenograft, Cell culture

1 Introduction

It has long been noted that developmental programs reemerge during cancer and tumors can be considered aberrant organs. Moreover, many malignant tumors have been histologically characterized as embryonic or poorly differentiated in nature. The embodiment of these observations has been the cancer stem cell (CSC) hypothesis that can be traced back to nearly 50 years ago [1]. Dr. John Dick and colleagues provided direct experimental evidence for a population of self-renewing, highly tumorigenic CSCs in the context of leukemia in 1997 by utilizing CD34+ cells enriched from human patients [2]. Subsequent studies provided similar experimental evidence for human solid tumors including breast [3], colon [4, 5], prostate [6, 7], and brain [8–11]. These pioneering studies made use of differential cell surface marker expression to enrich for CSCs and compare their phenotypes to non-stem tumor cells (NSTCs) [12]. Given the importance of CSCs in tumor growth and therapeutic resistance [13–15], the isolation and characterization is crucial to understanding the phenotype of the subpopulation. To facilitate these investigations, methods including Magnetic-activated Cell Sorting (MACS) and

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Fluorescence-activated Cell Sorting (FACS) were developed and adapted from previous methods used to identify various hematopoietic and immunological cell populations [16]. Next-generation strategies to enrich CSCs are being developed based on reporter systems and allow for the real-time assessment of the CSC state. These reporter systems utilize fluorescent gene transduction that allows for the immediate quantification of stem cell delineating proteins, such as NANOG, SOX2, and OCT4 [17, 18]. In this chapter, we will provide the necessary information required to isolate and culture CSCs and their non-stem tumor cell counterparts.

2 Materials

Note: All cell culture should be performed inside a tissue culture hood to maintain sterility.

2.1 Isolating Cells from Tissue

1. Primary tissue or patient-derived xenograft.
2. Worthington Papain Dissociation System (4 vials).
   (a) Vial 1—Sterile Earle's Balanced Salt Solution (EBSS) with bicarbonate and phenol red.
   (b) Vial 2—Papain containing L-Cysteine and EDTA.
      i. Reconstitute with 5 mL of Vial 1 and incubate for at least 10 min.
   (c) Vial 3—Deoxyribonuclease I (DNase).
      i. Reconstitute with 500 μL of Vial 1.
   (d) Vial 4—Ovomucoid protease inhibitor with bovine serum albumin.
      i. If new, reconstitute with 32 mL of Vial 1. Proceed to **step ii.**
      ii. If already reconstituted, take 5.3 mL and place in 15 mL conical (Vial 5).
3. 70 % ethanol.
4. 10 cm petri dish.
5. Autoclaved scissors, forceps, and razor blade.
6. Neurobasal Complete Media (NBMc): 500 mL Neurobasal Media (without Phenol Red), 10 mL B27, 50 μL FGF, 20 μL EGF, 5 mL Sodium Pyruvate, 5 mL l-Glutamine, 5 mL Penicillin and Streptomycin.
7. Neurobasal Null Media (NBL): 500 mL Neurobasal Media (without Phenol Red), 5 mL Penicillin and Streptomycin.
8. Sterile H_2O.
9. Sterile Dulbecco's Phosphate Buffered Saline (D-PBS).
10. Two, 50 mL conicals.

11. Two, 15 mL conicals.
12. Three, 70 micron cell strainer.
13. Red Biohazard bag (small).

2.2 Sorting

2.2.1 MACS Sorting by CD133

1. CD133 microbeads.
2. MACS cell separation column.
3. LS column (w/plunger).
4. FcR blocking reagent.
   (a) Dilute MACS BSA stock solution 1:20 with autoMACS® Rinsing Solution. Store at 4 °C.
5. 40 μm cell strainer.
6. NBMc.
7. 50 and 15 mL conical tubes.
8. 10 % FBS DMEM containing penicillin and streptomycin.

2.2.2 FACS Sorting

1. Surface protein primary antibody (e.g., CD133).
2. DAPI (for viability staining).
3. NBL.
4. D-PBS.
5. FACS buffer.
   (a) 2 parts BSA Bovine Serum Albumin (BSA).
   (b) 1 part NBL.
6. Two, 40 μm cell strainer.
7. Four, 15 mL conicals.
   (a) One, 15 mL conical containing 10 mL NBMc.
   (b) One, 15 mL conical containing 10 mL 10 % FBS DMEM P/S.

2.2.3 GFP-Reporter System Sorting

1. Bulk cultured cells.
2. Fluorescent lentivirus (e.g., NANOG-GFP).
3. NBMc and 10 % DMEM.
4. Disposable serologic pipette.
5. 20 % bleach.
6. T-25 and T-75 flasks.
7. Three, 15 mL conicals.
8. One, 40 μL cell strainer.

2.3 Expansion

2.3.1 Sorted Culture

1. Cancer Stem Cell (CSC) culture.
   (a) Petri Dish (any size).
   (b) Neurobasal Complete Media (NBMc) (*see* **Notes 1** and **2**).

(c) Accutase.

(d) D-PBS.

2. Non-stem Cancer Cell (NSTC) culture.
   (a) Tissue-culture-treated plate (adherent).
   (b) 10 % DMEM Media: 500 mL DMEM, 50 mL Fetal Bovine Serum (FBS), 5 mL Penicillin and Streptomycin (P/S).
   (c) Trypsin.
   (d) D-PBS.

2.3.2 Bulk Culture

1. Same Materials as CSC Culture

2.3.3 Basement Membrane Culture

1. Membrane preparation.
   (a) Cold NBL.
   (b) Concentrated Geltrex.
2. Culture materials.
   (a) Filtered Flask (any size).
   (b) Membrane solution.
   (c) NBMc.
   (d) Accutase.
   (e) D-PBS.

3 Methods

3.1 Isolating Cells from Tissue (See Notes 3 and 4)

1. After retrieving tumor sample, store on ice until ready to dissociate.
2. Sanitize cell culture hood with 70 % ethanol and gather autoclaved instruments.
3. Spray vials with 70 % ethanol before bringing into hood.
4. Place tumor into prepared petri dish with D-PBS.
5. Remove D-PBS; wash twice with 10 mL of D-PBS.
6. Mince tumor using autoclaved razor blade.
7. Add 350 μL of **vial 3** to **vial 2** and apply mix to tumor. Continue mincing and triturating to achieve slurry-like consistency.
8. Place dish in an incubator for 1 h.
9. Prepare two 50 mL conicals with 70 μL filters.
10. Transfer slurry to first 50 mL conical. Rinse the dish with D-PBS and add the wash to the slurry.
11. Triturate the filtered slurry and repeat the process into the second 50 mL conical. Rinse the first conical with D-PBS and transfer it to the second conical.

12. Centrifuge at 200 × *g* for 5 min.
13. While centrifuging, add 2.7 mL of **vial 1** and 300 μL of **vial 5** to a 15 mL conical. Add 150 μL of **vial 3** to the conical.
14. Remove supernatant and gently resuspend pellet in solution from **step 13.**
15. Transfer cell suspension to **vial 5** but do not mix! Layer the suspension on top and then centrifuge at 75 × *g* for 6 min (*see* **Note 5**).
16. Remove the supernatant and resuspend cells in 10–20 mL of NBMc. Incubate overnight at 37 °C.
17. Centrifuge at 200 × *g* and remove supernatant.
18. *Set centrifuge to 315 × g for 3 min, it is important to centrifuge immediately following this step*. Resuspend in 10 mL of D-PBS. Add 30 mL of sterile H_2O to the tube, invert *immediately* to mix. Centrifuge *immediately*.
19. Proceed to sorting protocol of your choice.

3.2 Sorting

3.2.1 MACS Sorting by CD133

Magnetic Labeling

1. Resuspend in NBMc and prepare single cell suspension (*see* **Note 6**).
   (a) Strain cells using a 40 μm cell strainer.
   (b) Spin cells at 200 × *g* for 5 min.
   (c) Remove supernatant and wash the cells with NBL.
   (d) Count cells and resuspend up to 30 million cells in 300 μL of NBL.
2. Add 100 μL of FcR Blocking Reagent per 30 million cells (*see* **Note 7**).
3. Label cells with 100 μL of CD133 microbeads per 30 million cells (final volume of 500 μL per 30 million cells) (*see* **Note 8**).
4. Wash cells by adding 1–2 mL of buffer and centrifuge at 200 × *g* for 10 min. Aspirate supernatant completely.
5. Resuspend the cell pellet in 500 μL of NBL per ten million cells (*see* **Note 9**).

Magnetic Separation

1. Place column in the magnetic field of a MACS Separator. Place a 50 mL conical tube under the column to collect the CD133– cells.
2. Label a 15 mL conical tube for CD133+ cells.
3. Wet the column by rinsing with 3 mL of NBL.
4. Wash the column with 1 mL NBL and collect flow-through in the same tube as the prior step.

5. Place a fresh 50 mL conical tube under the column and wash the column three times with 3 mL NBL (Do not keep these cells for culture).
6. Apply cell suspension and collect flow-through (unlabeled cells) (*see* **Note 10**).
7. Remove column from separator.
   (a) Rinse column two times with 5 mL NBL and flush out magnetically labeled cells using a plunger into the CD133+ conical tube from **step 2b.**

Cell Culture

1. Spin down the negative cells (to remove all NBL), count, and plate in DMEM + serum + pen/strep in 10 cm tissue culture-treated dishes.
2. Count CD133+ fraction and plate in NBMc containing EGF and bFGF growth factors in non-tissue culture-treated dishes (one million cells/10 cm dish).

3.2.2 FACS Sorting

1. Resuspend your newly dissociated cells in FACS buffer based on size of pellet.
2. Triturate suspension and filter through 40 μL strainer into 15 mL conical to ensure single cell suspension.
3. Count and dilute to 5×10^6 to 1×10^6 cells per mL, then stain with CD133 antibody at a ratio of 1:20.
4. Incubate at room temperature for 45 min on a shaker.
5. Add 3 mL of NBL to conical, then spin at 200 × *g* for 5 min.
6. Resuspend in NBL, count cells, and dilute to $2–3 \times 10^6$ cells per mL.
7. Triturate suspension and again filter through a 40 μL strainer to ensure single cell suspension for sorting.
8. 10 min before sorting, add DAPI solution to cells at 10 ng/1 mL.
9. Bring cell solution along with one 15 mL conical of each media type (NBMc and DMEM) to sorting for bulk collection.
10. After sorting, spin your sorted populations and plate in appropriately sized plates. Size will vary based on initial tumor size/density as well as FACS efficiency.

3.2.3 GFP-Reporter System Sorting (See Note 11)

1. Culture bulk cells in adherent conditions following basement membrane culture protocol (*see* Subheading 3.3.3).
2. Aspirate media from adherent bulk culture cells when confluency reaches ~30 % (*see* **Note 12**).
3. Using a disposable serologic pipette, add minimum effective MOI μL of lentivirus to 3 mL of NBMc. This will change

depending on your particular virus, refer to provider for concentration. Add this mixture to the flask. Return pipette to sleeve and allow to sit in 20 % bleach for 15 min (*see* **Note 13**).

4. Allow culture to sit overnight. Add 3 mL of NBMc to the flask, bringing culture volume to 6 mL.
5. Split cells when confluent and plate in a T-75.
6. Allow cells to grow to confluency. Split and count cells. Dilute cell suspension to 3×10^6 cells per mL.
7. Triturate suspension and filter with 40 μL strainer.
8. Label with DAPI at 10 ng/1 mL.
9. Prepare two 15 mL conicals for sorting. One with 5 mL NBMc, and one with 5 mL 10 % DMEM.
10. Sort for top 5 % of GFP positive cells, plate into appropriate size flask after sorting based on number of cells.

3.3 Expansion

3.3.1 Sorted Culture [19]

1. CSC Culture (*see* **Note 14**).
   (a) CSCs plated in a petri dish with NBMc will grow in suspension, forming spheres [7]. Sphere formation is property previously reported to be defining for the in vitro growth of neural stem cells [20] (*see* **Note 15**).
   (b) Collect media and place into appropriate size conical. Spin for 5 min at 200 × *g* (*see* **Note 16**).
   (c) Aspirate supernatant and resuspend pellet in 1 mL of Accutase. Place this suspension in an incubator at 37°C for 5 min.
   (d) Following incubation, inactivate Accutase using a 1:1 dilution with D-PBS.
   (e) Spin for 5 min at 200 × *g*.
   (f) Aspirate supernatant, resuspend in NBMc, and replate as desired. Avoid plating more than 1:2 or less than 1:20 in a single petri dish.
2. CSC Culture 2 [21].
   (a) Transfer spheres from dish to a conical tube using a serological pipette (*see* **Note 17**).
   (b) Allow the spheres to sediment in the conical tube for 2–5 min (*see* **Note 18**).
   (c) Remove media leaving the sedimented cells in the tube.
   (d) Add 10 mL of D-PBS without calcium chloride and magnesium chloride (Sigma D8537) to the cells and incubate at room temperature for at least 10 min.
   (e) Resuspend/triturate the cells 6–7 times until no cell clumps are visible (*see* **Notes 19** and **20**).

(f) Spin down the cells at 200 × *g* for 5 min and plate cells at desired cell density based on the size of the spun-down pellet.

3. NSTC culture.
   (a) Non-stem tumor cells are grown in adherence, and should be split when dish reaches 80–90 % confluency. Size of dish dictates amount of media. 10 cm dishes require 10 mL of media, while 15 cm dishes require 20–25 mL.
   (b) Aspirate media and wash dish with 5–10 mL of D-PBS to promote detachment. Aspirate D-PBS.
   (c) Cover dish with enough Trypsin to completely and evenly coat cells. Place dish in an incubator at 37 °C for 5 min.
   (d) Following incubation, inactivate Trypsin with 1:1 dilution of DMEM 10 % FBS.
   (e) Move suspension to conical and spin for 5 min at 200 × *g*.
   (f) Aspirate supernatant, resuspend pellet in DMEM, and replate as desired. Avoid plating more than 1:2 or less than 1:10 in a single dish.

3.3.2 Bulk Culture

If you do not sort for stem cell markers, bulk tumor cells may be maintained in suspension following CSC protocol (*see* Subheading 3.3.1) or in adherence following basement membrane protocol (*see* Subheading 3.3.3).

3.3.3 Basement Membrane Culture

1. Membrane Preparation.
   (a) Geltrex is to be thawed on ice for 30 minutes (or until thawed) or in a 4 °C overnight.
   (b) Once Geltrex is finishing thawing, remove NBL from 4 °C and place desired volume in conical.
   (c) Solution is prepared using 1.6–2 μL of Geltrex for every 1 mL of NBL.
   (d) When ready, pull cold media into pipette tip to prevent premature polymerization of Geltrex.
   (e) Quickly transfer desired volume of Geltrex into conical, tightly close conical, and invert to mix. If done properly, no strands of Geltrex will be visible in solution.
   (f) Store in 4 °C until ready to coat flasks.
      i. Coat flasks overnight in an incubator. Coated flasks are viable for 1–2 months (*see* **Note 21**).
2. Culture Protocol.
   (a) Useful for either bulk culture or sorted CSCs, membrane culture flasks should be split when approaching 80–90 %

confluency. Size of flask dictates amount of media. T-25 flasks require 6 mL of media, while T-75 flasks require 10 mL.

(b) Aspirate media and wash dish with D-PBS to promote detachment. Aspirate D-PBS.

(c) Cover flask with enough Accutase to completely and evenly coat cells. Place dish in an incubator at 37 °C for 5 min.

(d) Following incubation, inactivate Trypsin with 1:1 dilution of D-PBS 10 % FBS.

(e) Move suspension to conical and spin for 5 min at 200 × *g*.

(f) Aspirate supernatant, resuspend pellet in NBMc, and replate as desired. Avoid plating more than 1:2 or less than 1:10 in a single flask.

4 Notes

1. Store all cell culture components (media, Trypsin/Accutase, D-PBS) at 4 °C. Before use, they should be warmed in a 37 °C water bath for 5–10 min, or until the bottle is no longer cold to the touch. Do not overheat; this can cause the solutions to become ineffective.
2. If stored at 4 °C, NBMc and DMEM both will stay usable for 2–4weeks. Different volumes should be prepared based on need. Maintain the same ratio and concentrations of products.
3. Protocol is included with Worthington dissociation kit.
4. When working in a tissue-culture hood, keep your gloves sterile by spraying with 70 % Ethanol before bringing hands into the hood. Avoid crossing over any open bottles or flasks.
5. There will be two distinct layers in the supernatant following centrifugation. Membrane fragments collect at the point of contact between the two layers, while dissociated cells pellet at the bottom of the conical.
6. If trypsinizing cells the same day, let cells recover for at least 5 h prior to sorting as cell dissociation cleaves the CD133 antigen.
7. Avoid light exposure when adding blocking reagent and microbeads to cells.
8. Agitate the tube every 5–10 min to ensure adequate mixing.
9. Use a maximum of 30 million cells per separation column.
10. Send first pass CD133-cells through a new column and keep only the cells that initially pass through the column.
11. The process of lentiviral transduction of a fluorescent reporter system spans a few days. Be sure to plan your timing

appropriately. You will also need an uninfected control for FACS sorting by GFP.

12. We find that lentiviral transduction is most efficient when cells are at ~30 % confluency.
13. When using a pipette to triturate or transfer cell-containing media, avoid introducing bubbles. This can be achieved by not triturating to rigorously and by not continuing to pull solution into the pipette when there is not enough remaining in the dish. Bubbles can be detrimental to cell health and survival.
14. The virus can be premixed into the media in a 15 mL conical or added directly to culture. If you are having troubles with transduction, try premixing.
15. Size of petri dish dictates amount of media. 10 cm dishes require 10 mL of media, while 15 cm dishes require 20–25 mL. When the dish becomes crowded, the spheres begin appearing dark in the middle, or single cells become common, it is time to split.
16. $200 \times g$ is how fast we centrifuge our samples, but there is a range of acceptable speeds and lengths. Cells can be centrifuged at as slow as $85 \times g$ and for as short as 3 min while still obtaining a usable pellet.
17. Use D-PBS to wash lightly adhering spheres from the dish.
18. If cells are of a low density, cells may be spun down at $200 \times g$ for 2–3 min.
19. Pre-wet the serological pipette (cells may stick to the pipette) by transferring 6–7 mL from the top layer of D-PBS to a new tube. Use the same pipette to resuspend the cells.
20. This step may be repeated one additional time or unbroken cell clumps may be left behind in the original tube while the single cell suspension is transferred to another tube. In this scenario, only the single cell suspension will be used for culturing.
21. Before plating any cells, remove Geltrex solution and wash with same volume of D-PBS.

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Chapter 11

Cardiac Progenitor Cell Extraction from Human Auricles

Paolo Di Nardo and Francesca Pagliari

Abstract

For many years, myocardial tissue has been considered terminally differentiated and, thus, incapable of regenerating. Recent studies have shown, instead, that cardiomyocytes, at least in part, are slowly substituted by new cells originating by precursor cells mostly embedded into the heart apex and in the atria. We have shown that an elective region of progenitor cell embedding is represented by the auricles, non-contractile atria appendages that can be easily sampled without harming the patient. The protocol here reported describes how from auricles a population of multipotent, cardiogenic cells can be isolated, cultured, and differentiated. Further studies are needed to fully exploit this cell population, but, sampling auricles, it could be possible to treat cardiac patients using their own cells circumventing rejection or organ shortage limitations.

Key words Heart, Cardiac progenitor cells, Differentiation, SCA-1, Immunomagnetic separation

1 Introduction

Innumerable experimental studies and clinical trials on stem cell potential to repair the injured myocardium have generated frustrating results, since only a minor number (<3 %) of injected cells [1] home into the recipient myocardium and their contribution to the heart performance is, at least, questionable [2, 3]. Indeed, many aspects of the procedure to isolate, expand in vitro, and implant stem cells in the recipient organ clearly show drawbacks that the enthusiastic irrational approach of the early cell therapy protocols has missed to clarify. Among others, the lack of standardization has been a major cause of fault of current protocols. A plethora of cells, sometimes isolated from the same tissue, have been claimed to be optimal for heart repair without unambiguous evidence. The different stem/progenitor cells populations endowed with some cardiogenic potential so far identified into the myocardium are, very likely, representative of the differentiating steps of a single process. Therefore, it is necessary to define stringent protocols to isolate a proper progenitor cell population. This population might be non-representative of the very early

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ancestor cells, but it can be constituted of cells in an early stage of differentiation still retaining sufficient potential to generate cell types needed to repair the myocardium. Such characteristics have been initially identified in cells resident into the myocardium and expressing c-kit (or CD117). However, the expression of c-kit has shown to be unstable [4] and, after enrichment, to rapidly decline representing no more than 15 %–20 % of cell population, while the stemness marker SCA-1 (Stem Cell Antigen 1) was stably expressed in most cells and uphold for more than 50 passages. This could imply that c-kit^{pos} and SCA-1^{pos} progenitor cells are not different cell populations, but different phenotypic pools of the same original population in which c-kit expression identifies a more immature cell stage and SCA-1 a more mature, actively growing and potentially cardiogenic cell stage. SCA-1^{pos} cells can be isolated without additional risks for the patient from the auricles, an appendage of mammalian atria secreting large amounts of growth factors (e.g., atrial natriuretic peptide, etc.), but irrelevant to the overall contractile function.

Very crucial is the procedure to isolate and culture SCA-1 progenitor cells. In fact, cell viability/function is heavily downrated under current protocols that neglect the stem cell peculiar environment (niches) in vivo [5]. Cell therapy is aimed at replacing permanently injured cells with functional myocardial cells (not only cardiomyocytes) to reconstitute the native myocardial texture and architecture, and to achieve a (quasi)physiological heart performance. In this vision, the protocol here reported allows to isolate human cardiac progenitor cells with a stable phenotype, but prone to differentiate toward a cardiomyocyte phenotype when cultured in a proper environment [6, 7]. However, it must be considered that a proper in vitro environment for cardiac progenitor cells is far to be defined: as soon as the knowledge about stem cell behavior improves, the possibility of preserving stem cell full potential will increase and cardiac cell therapy will be easily and safely applied in the clinical setting.

2 Materials

2.1 Reagents

1. Dulbecco's Phosphate Buffered Saline (D-PBS) without calcium chloride and magnesium chloride.
2. Trypsin 0.05 %—EDTA 0.02 % Solution 1×.
3. Collagenase Type II—1500 U.
4. Leibovitz medium L-15.
5. Dulbecco's Modified Eagle medium high glucose.
6. Fetal Bovine Serum (FBS).
7. Penicillin 10,000 I.U./mL—Streptomycin 10 mg/mL (PEN-STREP) added to culture medium for a final concentration of 100 I.U./mL penicillin and 100 (μg/mL) streptomycin.

8. L-glutamine solution added to culture medium at a final concentration of 2 mM.
9. Retinoic Acid (RA) at a final concentration of 300 ng/mL.
10. Insulin-like Growth Factor-1 (IGF-1).
11. Linoleic acid.
12. Vascular Endothelial Growth Factor (VEGF).
13. Gelatin type A powder.
14. CD117 (c-kit) MicroBeads (Catalog Number 130-091-332; Miltenyi Biotech).
15. Anti-Stem cell antigen 1 (Sca-1) Microbead Kit (FITC) (Catalog Number 130-092-529; Miltenyi Biotech).
16. Anti-CD117-PE conjugate antibody (Catalog Number 130-091-734;Miltenyi Biotech).
17. Bovine serum albumin.
18. EDTA solution.
19. Fibronectin.
20. Milli-Q Water.

2.2 Equipment

1. Scissors, tweezers, and sterile razor blades.
2. 15 mL polypropylene, conical bottom, sterile centrifuge tubes.
3. 50 mL polypropylene, conical bottom, sterile centrifuge tubes.
4. 5 mL pipettes.
5. 10 μL and 1000 μL sterile tips.
6. 1.5 mL microcentrifuge tubes.
7. 5 mL syringes.
8. 100 μm cell strainers.
9. 0.20 μm sterile filters.
10. 40 μm sterile cell strainers.
11. Six-well cell culture plates.
12. Culture dishes 100 mm.
13. MiniMACS Separator.
14. MACS MultiStand (Catalog Number 130-042-303; Miltenyi Biotech).
15. MS Columns (Catalog Number 130-042-201; Miltenyi Biotech).
16. MACS Acrylic Tube Rack (Catalog Number 130-041-046; Miltenyi Biotech).
17. MACSmix Tube Rotator (Catalog Number 130-090-753; Miltenyi Biotech).
18. 5 mL round-bottom polystyrene tubes.

19. Laminar Flow Cabinet.
20. CO_2 incubator humidified, 37 °C, 5 % CO_2.
21. Centrifuge.
22. Water bath.
23. Pipette-aid.
24. Inverted light microscope.

3 Methods

Carry out all the procedures under a Flow Laminar Cabinet unless indicated otherwise.

3.1 Reagent Setup

1. Reconstitute Collagenase Type II—1500 U with 5 mL of L15 media to obtain a solution of 300 units/mL and store at 2–8 °C.
2. Dissolve RA in DMSO (3 mg/mL solution) and store the stock solution of 0.01 M in light-protected vials at −20 °C; add with tissue culture medium right before use at a final concentration of 300 ng/mL.
3. Dissolve IGF-1 powder in sterile water to a concentration of 1.0 mg/mL and freeze at −20 °C in working aliquots; use at a final concentration of 0.1 ng/mL in complete culture medium.
4. Use linoleic acid at a final concentration of 0.8 μg/mL in complete culture medium.
5. Reconstitute VEGF in sterile water to a concentration of 0.1–1.0 mg/mL, and store in appropriate aliquots at −20 °C; use at a final concentration of 0.1 ng/mL in complete culture medium.
6. Prepare Cell Extraction Medium adding in 440 mL of DMEM: 50 mL of FBS, 5 mL of PEN-STREP, and 5 mL of L-Glutamine. Store at 2–8 °C.
7. Prepare Complete Culture Medium in 50 mL polypropylene sterile tube adding in cell extraction medium (DMEM 10 % FBS, PEN-STREP, and L-Glutamine, as prepared in **step 6**): 300 ng/mL of RA, 0.1 ng/mL of IGF-1, 0.8 μg/mL of linoleic acid, 0.1 ng/mL of VEGF. Store the medium protected from light at 2–8 °C.
8. Prepare 0.1 % Gelatin dissolving 0.5 g of gelatin type A (Sigma) in 500 mL of MilliQ water and mix. Autoclave at 121 °C for 15 min to sterilize and dissolve the gelatin. After cooling, store at 2–8 °C.
9. Coat 6-well plates or 100 mm culture dishes with 2 mL/well or 5 mL/dish of 0.1 % gelatin; incubate the plate for 30 min at 37 °C and remove the exceeding solution before using.

10. Dissolve Fibronectin in MilliQ water 1 mg/mL and store working aliquots at −20 °C.
11. Prepare fibronectin pre-coated 6-well plates by diluting fibronectin stock solution in sterile D-PBS (2 μg/mL) and coat the wells with a minimal volume. Put the plates in the incubator for 1 h, then remove the excess, and use the plates or store them sealed with parafilm at +4 °C in a sterile container up to 1 month.
12. Prepare a stock solution of 0.5 M EDTA dissolving 14.6 g EDTA powder in 500 mL MilliQ water. Autoclave at 121 °C for 15 min and store a 2–8 °C.
13. Buffer for immunomagnetic separation: prepare a solution containing D-PBS, pH 7.2, 0.5 % bovine serum albumin, and 2 mM EDTA. Filter the solution through a 0.2 μm filter and keep at 2–8 °C.

3.2 Collecting the Samples

1. Receive the auricle fragments in cold sterile D-PBS (*see* **Note 2**) (Fig. 1a).
2. Wash twice and prefund the fragments with cold D-PBS using a syringe to remove residual blood.
3. Place the fragments on a not-coated 100 mm culture dish in cold D-PBS and on ice.
4. Cut the samples into small pieces (1–2 mm^3) using scissors, tweezers, and razor blades.

3.3 Trypsin Digestion of the Samples

1. Carefully remove the D-PBS by aspiration and add trypsin-EDTA for digestion. For 1–3 cm^3 of starting specimens, a volume of 5 mL of Trypsin solution is enough to digest the fragments obtained and kept in a not-coated 100 mm culture dish (*see* **Note 3**).

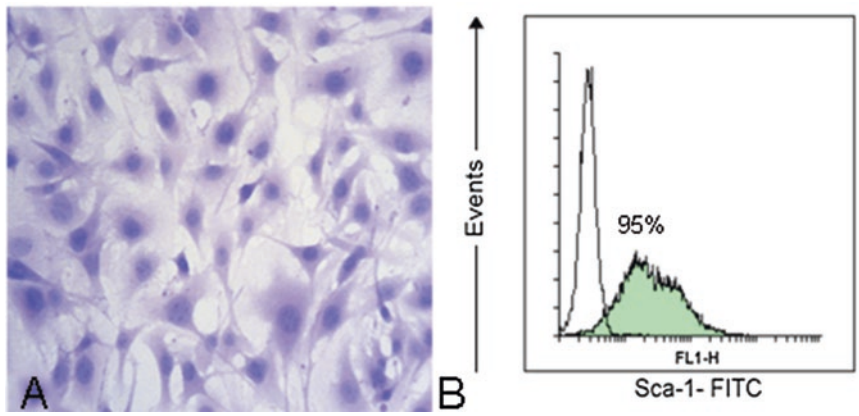

Fig. 1 (**a**) Hematoxylin and eosin staining of cardiac progenitor cells after 10 days in culture (Magnification 20×); (**b**) Flow cytometry analysis of SCA-1 expression in CPCs

2. Pipet up and down the solution and incubate at +4 °C for 2 h while gently rotating (*see* **Note 4**).
3. After digestion, aseptically transfer the fragments in a 50 mL conical tube, add a double volume, at least, of pre-warmed Cell Extraction Medium to inactivate the trypsin enzyme by repeatedly pipetting up and down with a 10 mL pipette.

3.4 Collagenase Digestion

1. After digestion, put the sample in a water bath at 37 °C for 3–4 min.
2. Remove the sample from the water bath and add Collagenase II in L15 medium. For a volume of 15 mL of tissue volume, use 2 mL of Collagenase from stock solution.
3. Incubate the solution at 37 °C in a water bath for 30–40 min and shake it every 10 min to make the digestion homogeneous.

3.5 Sample Filtering and Plating

1. Centrifuge the sample at 280 ×*g* for 5 min, so as to allow the fragments to settle on the bottom.
2. Place a 100 μm cell strainer on a sterile 50 mL conical tube and equilibrate with 5 mL Complete Culture Medium; collect this volume and use to resuspend the fragments (see below).
3. After centrifugation, carefully discard the supernatant under the cabinet. If the fragments did not settle, centrifuge other 3 min, then aspirate the supernatant using a 5 mL pipette (*see* **Note 5**).
4. Resuspend the pellet in 5 mL of collected Cell Culture Medium and pipet the solution into the cell strainer.
5. Transfer the fragments retained on the filter in a 0.1 % gelatin pre-coated culture plate and add small volume of Complete Culture Medium (500 μL for 10 cm^2) (*see* **Notes 6** and **7**) (Fig. 1b).
6. Incubate the plate at 37 °C in a CO_2 humidified incubator for 24 h. The next day, observe fragment attachment under an inverted light microscope. If they adhere to the surface, add carefully Complete Culture Medium and recover the sample in the incubator. The following day, replace the medium and nonadhering fragments with fresh Complete Culture Medium, which is then changed every second day (Fig. 1a).

3.6 Cell Culturing

After 10–15 days, fibroblastoid cells migrate from the fragments until reaching 80–90 % confluence.

1. Aspirate the medium and wash the cells with pre-warmed D-PBS.
2. Remove the D-PBS and add trypsin-EDTA solution (500 μL/well in a 6-well plate or 2 mL in a 100 mm culture dish).

3. Incubate at 37 °C in a CO_2 humidified incubator for few minutes until to completely detach cells. Check the progression under the microscope.
4. Once cells are detached, add at least a double volume of Complete Culture Medium and gently pipet up and down to be sure that cell clumps are disaggregated into single cells (*see* **Note 8**).
5. Transfer in a sterile 50 mL tube and centrifuge at 200 ×*g* for 5 min.
6. Discard the supernatant and resuspend in an adequate volume of fresh Complete Culture Medium.
7. Pass cell suspension through a 40 μm cell strainer to remove completely any residual tissue fragment.
8. The first time, split cells in a 1:2 ratio in a new not-coated 6-well plate (*see* **Note 9**).
9. Once cells reach 80–90 % confluence (2–3 days), detach them again following the previous steps.
10. Resuspend cell suspension in fresh Complete Culture Medium and determine cell number. If you have almost 10^7 cells, proceed with the following steps; otherwise, let the cells grow until reaching an adequate number.

3.7 Immunomagnetic CD117 Positive Cells Selection

1. Centrifuge cell suspension, aspirate supernatant, and resuspend upto 10^6 cells in 300 μL of cold buffer and transfer in a 1.5 mL sterile microcentrifuge tube.
2. Add 100 μL of FcR Blocking Reagent (Miltenyi Biotech) and 100 μL of anti-CD117 microbeads, mix and incubate for 15 min at +4 °C in the dark while rotating on a tube rotator (*see* **Note 10**).
3. Add 1 mL buffer, centrifuge at 300 ×*g* for 10 min and remove carefully supernatant.
4. Resuspend cells in 500 μL of cold buffer.
5. Place a MS MACS Column in a MACS separator containing a magnet and attach to a MultiStand. Under the column, place a tube rack with 1.5 mL sterile tubes. Wash the column with 500 μL of cold buffer to equilibrate.
6. Transfer cell suspension onto the column and let the solution pass through it in the magnetic field without using the plunger.
7. Collect negative cells in a new 1.5 mL sterile tube and wash twice the column with 500 μL of cold buffer without removing it from the separator. Here, you get the $CD117^{neg}$ fraction of cell population (*see* **Note 11**).
8. Remove the column from the separator and place it into a new sterile 1.5 mL tube.

9. Add 500 μL of cold buffer, insert the supplied plunger in the column, and apply a gentle pressure to flush out the CD117^{pos} fraction of cell population.
10. Wash the column twice with 500 μL of cold buffer and collect every eluate (*see* **Note 12**).
11. Plate CD117^{pos} cells on a fibronectin pre-coated 6-well plate at a density of 5 × 10^3 cells/cm^2 in Complete Culture Medium.

3.8 Immunomagnetic SCA-1 Positive Cell Selection

After growing CD177^{pos} cells up to reach at least 10^7 cells, carry out the immunomagnetic selection of SCA-1 positive cells.

1. Detach cells according to the steps described in Subheading 3.6.
2. Centrifuge cell suspension, completely remove supernatant, and resuspend up to10^6 cells in 90 μL of cold buffer; transfer in a 1.5 mL sterile microcentrifuge tube.
3. Add 10 μL of anti-SCA-1 FITC antibody, mix and incubate for 10 min at +4 °C in the dark while rotating on a tube rotator (*see* **Note 10**).
4. Add 1 mL buffer and centrifuge at 300 × *g* for 10 min.
5. Aspirate the buffer, add 80 μL of new cold buffer and 20 μL of Anti-FITC microbeads. Mix and incubate for 15 min at +4 °C in the dark while rotating on a tube rotator (*see* **Note 11**).
6. After, add 1 mL of cold buffer, centrifuge again at 300 × *g* for 10 min, and remove carefully supernatant.
7. Resuspend cells in 500 μL of cold buffer.
8. Place a MS MACS Column in a MACS separator and proceed as described in Subheading 3.7, **steps 5–7**. Now you have the unlabeled SCA-1^{neg} fraction of cell population. Keep this fraction for immunophenotype characterization (*see* Subheading 3.9) (*see* **Note 12**).
9. Remove the column from the separator, place it into a new sterile 1.5 mL tube, and add 500 μL of cold buffer.
10. Insert the plunger in the column and flush out the SCA-1^{pos} fraction of cell population.
11. Wash the column twice with 500 μL of cold buffer and collect every eluate (*see* **Note 13**). Since the SCA-1^{pos} subpopulation is conjugated with FITC fluorochrome, it is ready for flow cytometry immunophenotyping, thus take 1.2 × 10^6 cells and follow the next step (*see* Subheading 3.9). Plate the remaining part of the Cardiac Progenitor Cells (CPCs) according to your experiments (*see* **Note 14**).

3.9 Immunophenotype Characterization

For CD117^{pos} cell characterization:

1. After growing CD117^{pos} cells, detach cells from the plate according to the Subheading 3.6, **steps 1–5**.

2. Remove buffer by centrifuging at 300 ×*g* for 10 min.
3. Resuspend 1×10^6 positive cells in 1 mL D-PBS and divide into two 1.5 mL tubes (500 μL/tube).
4. Incubate one aliquot of cells with 10 μL of anti-CD117-PE for 5 min in the refrigerator.
5. Wash the samples twice with ice-cold PBS, fix them in buffered 1 % PFA, 2 % FBS for 15 min at +4 °C, and analyze in a flow cytometer within 48 h.
6. Alternatively, remove PFA by centrifuging twice in ice-cold PBS for 5 min at 300 ×*g*, resuspend in fresh D-PBS, and transfer in polypropylene tube for flow cytometry.

   For $CD117^{pos}SCA\text{-}1^{pos}$ cell characterization, after SCA-1 positive selection (*see* Subheading 3.8, **step 11**):
7. Centrifuge $SCA\text{-}1^{pos}$ cell subpopulation conjugated with FITC fluorochrome at 300 ×*g* for 10 min.
8. Resuspend 1×10^6 positive cells in 1 mL D-PBS and divide into two 1.5 mL tubes (500 μL/tube) (*see* **Note 15**).
9. For double staining, incubate one sample with 10 μL of anti-CD117-PE for 5 min in the refrigerator. Keep the other sample in ice-cold until the end of the procedure.
10. Wash the double-stained sample twice in ice-cold PBS for 5 min at 300 ×*g*, resuspend in D-PBS, transfer in polypropylene tubes, and analyze at a flow cytometer (Fig. 1b).

4 Notes

1. Collagenase medium is stable at 2–8 °C for days.
2. The auricle fragments should be kept at 2–8 °C and the processing must be made within 1 h of receipt.
3. The volume of Trypsin-EDTA depends on the amount of fragments, so make sure to use an adequate volume of solution, so that all the pieces are digested.
4. During incubation, wrap the plate with a layer of parafilm to prevent accidental spills, without hampering gas exchange.
5. The aspiration should be as gentle as possible to avoid tissue fragment loss.
6. Distribute the fragments as uniformly as possible on the plate and use 6-well plates or 100 mm culture dishes according to the amount of material obtained.
7. It is important to use small volume of culture medium to enable fragments to adhere on the plate surface.
8. Some tissue fragments are still in culture, thus during cell detachment try to remove remaining pieces as much as possible.

9. Make sure that the seeding density will be not too low for the cells so that it may hinder cell survival or too high so that it may induce cell overgrowth.
10. Temperature and time are critical parameters in the immunomagnetic separation protocol. Both higher temperatures and longer incubation times may lead to unspecific cell labeling.
11. Avoid forming air bubbles while mixing, this may interfere with the labeling.
12. Add new buffer only after every elution.
13. The eluted fraction can be further purified passing it through another column by repeating the steps described above.
14. CPCs can be cryopreserved by storing them in liquid nitrogen in complete Culture Medium with 10 % DMSO.
15. Use a aliquot of not unlabeled cells obtained from SCA-1^{pos} selection (*see* Subheading 3.8, **step 8**) as unstained control and stain another aliquot with anti-CD117-PE according to the **steps 1–5** of Subheading 3.9.

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Chapter 12

Isolation and Culture of Satellite Cells from Mouse Skeletal Muscle

Antonio Musarò and Silvia Carosio

Abstract

Skeletal muscle tissue is characterized by a population of quiescent mononucleated myoblasts, localized between the basal lamina and sarcolemma of myofibers, known as satellite cells. Satellite cells play a pivotal role in muscle homeostasis and are the major source of myogenic precursors in mammalian muscle regeneration.

This chapter describes protocols for isolation and culturing satellite cells isolated from mouse skeletal muscles. The classical procedure, which will be discussed extensively in this chapter, involves the enzymatic dissociation of skeletal muscles, while the alternative method involves isolation of satellite cells from isolated myofibers in which the satellite cells remain in their in situ position underneath the myofiber basal lamina.

In particular, we discuss the technical aspect of satellite cell isolation, the methods necessary to enrich the satellite cell fraction and the culture conditions that optimize proliferation and myotube formation of mouse satellite cells.

Key words Satellite cells isolation, Muscle primary culture, Cell culture, Muscle differentiation, Myogenic program

1 Introduction

One of the most important homeostatic properties of skeletal muscle is the capacity to regenerate in response to different physiopathologic stimuli, recapitulating many aspects of muscle development [1]. The dominant role in muscle regeneration is played by the muscle stem cells known as satellite cells [1, 2], which reside between the basal lamina and sarcolemma of myofibers and were described as "*dormant myoblasts that failed to fuse with other myoblasts and are ready to recapitulate the embryonic development of the skeletal muscle fibers when the main multinucleate cell is damaged*" [2]. Thus, satellite cells are mitotically quiescent until required for growth or repair. Satellite cells are activated in response to both physiological stimuli (such as exercise) and pathological conditions (such as injury and degenerative diseases) to generate a

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committed population of myoblasts that can either fuse with existing myofibers, repairing damaged muscle fibers, or alternatively fuse to each other to form new myofibers [1]. A small minority does not differentiate but instead reenters quiescence to maintain the stem cell pool. The possibility to isolate and culture satellite cells from different experimental models added new insight into the biology of muscle stem population and revealed potential alterations in the capacity to proliferate, self-review, and/or differentiate. Moreover, the discovery of molecular markers selectively expressed by satellite cells but not by muscle fibers has contributed to the characterization of these markers [1]. Quiescent satellite cells specifically express c-Met, M-cadherin, FoxK, Pax-3, Pax-7, NCAM, syndecan 3 and 4, CD34, caveolin-1, Sox 8, Sox 15, VCAM-1, integrin-α7, integrin-β1, calcitonin receptor (CTR), lamin A/C, emerin, Hey1, and Heyl. The relevant markers of proliferating satellite cells, which are silent in quiescent satellite cells, are desmin, Myf-5, MyoD, and PCNA. Once activated, satellite cell progeny can follow one of two fates depending on MyoD activity. Satellite cells may down-regulate MyoD and self-renew, alternatively, satellite cells maintain MyoD expression but down-regulate Pax7 and activate myogenin expression, thus committing to differentiation.

The transition from cell proliferation to differentiation involves the down-regulation of proliferative-associated genes and cell-cycle withdrawal with the activation of specific markers, including myogenin, neonatal isoform of myosin heavy chain (MyHC), slow-twitch skeletal muscle troponin T (Tnnt1), cardiac and slow-twitch skeletal muscle Ca2+-ATPase (Atp2a2), insulin-like growth factor-2 (Igf-2), fibroblast growth factor receptor 4 (Fgfr4), nicotinic cholinergic receptor alpha polypeptide 1 (Chrna1), and cardiac/slow-twitch skeletal muscle troponin C (Tncc) [1].

Thus, skeletal muscle is an attractive model to study the regulation of tissue-specific gene expression due to existence of in vitro cell culture systems that spontaneously fuse to form differentiated muscle fibers, activating a battery of muscle-specific genes.

Muscle primary cultures require special conditions for optimal growth and differentiation and in this chapter we will discuss the protocol to isolate satellite cells and the potential experimental limitations.

2 Materials

2.1 Solutions and Media

1. Collagen Type I: dissolve 10 mg of Collagen Type I in 10 mL 20 % Acetic Acid. Store at room temperature.
2. Penicillin/streptomycin solution 100×-solution 10,000 U/mL penicillin and 10 mg/mL streptomycin.

3. L-glutamine 200 mM, sterile filtered cell culture tested.
4. Dulbecco's Phosphate Buffered Saline (PBS) without calcium chloride and magnesium chloride.
5. Collagenase Type II. Dissolve 1.5 mg of collagenase Type II in 15 mL of PBS (0.1 mg/mL).
6. Collagenase/Dispase solution (Roche). Dissolve 15 mg of Collagenase/Dispase in 15 mL (1 mg/mL) of PBS without calcium and magnesium. Filter and use fresh.
7. Dulbecco's Modified Eagle's Medium supplemented with 50 U/mL penicillin and 50 μg/mL streptomycin.
8. Heat-inactivated horse serum.
9. Heat-inactivated foetal bovine serum.
10. HEPES 1 M pH 7.0–7.6 cell culture tested.
11. Hanks' balanced salt solution (HBSS).
12. Trypsin-EDTA.
13. Dimethyl sulfoxide.
14. Chick Embryo Extract (CEE) (MP-Biomedicals). Alternatively prepare CEE from chicken eggs as reported in Subheading 2.2.
15. 10 % Goat Serum in PBS.
16. Primary antibodies:

MyoD (Santa Cruz Biotechnology 1:100 diluted in PBS), Myogenin (1:50 diluted in PBS), Myosin (MF-20 Hybridoma Bank, 1:20 diluted in PBS).

17. Secondary antibody anti-mouse Alexa Fluor® 568 (Thermo Fisher Scientific).
18. Vectashield mounting medium with Hoechst.
19. β-D-arabinofuranoside add 0.3 mg per 100 mL of culture media.
20. Skeletal Muscle Dissociation Kit, mouse, and rat (Miltenyi).
21. Freshly prepared PEB buffer: dilute MACS BSA Stock Solution (Miltenyi) 1:20 with autoMACS® Rinsing Solution (Miltenyi).

2.2 Preparation of Chick Embryo Extract

1. Clean day 9–11 chicken eggs by wiping with ethanol. Harvest the embryo into a large Petri dish. Sacrifice the embryo and place in a 10 mL syringe.
2. Pass embryos twice through the 10 mL syringe. Collect the processed embryos in a 50 mL conical centrifuge tube.
3. Add an equal volume of HBSS. Triturate solution with a wide bore 25 mL pipette. Gradually reduce pipette size until solution is able to be drawn into 5 mL pipette.
4. Freeze solution overnight at −20 °C.

5. Thaw solution and continue trituration until the solution can be passed through a Pasteur pipette.
6. Centrifuge solution in a table top centrifuge at 3000 rpm for 30 min.
7. Discard pellet, remove supernatant, and freeze this solution in 2 mL aliquots; store at −20 °C.

2.3 Culture Media

1. Growth Medium (GM): DMEM supplemented with 20 % Horse Serum or 20 % Foetal Bovine Serum, Penicillin/Streptomycin (50 U/mL/50 mg/mL), L-Glutamine (4 mM), Hepes (10 mM), CEE (3 %).
2. Differentiation Medium: DMEM supplemented with Horse Serum (5 %), Penicillin/Streptomycin (50 U/mL and 50 μg/mL), L-Glutamine (4 mM), and Hepes (10 mM).

3 Methods

Two major methods have been developed to obtain isolated satellite cells. The classical procedure, which will be discussed extensively in this chapter, involves the enzymatic dissociation of skeletal muscles, while the alternative method involves isolation of satellite cells from isolated myofibers. Notably, cells prepared by enzymatic digestion of whole muscle tissue are likely to contain a heterogeneous population of precursor cells, such as myogenic cells derived both from the satellite cell niche and from other populations in the muscle interstitium and vasculature (Fig. 1). Nevertheless, different methods, such as differential centrifugation, preplating, Percoll gradient, Fluorescence Activated Cell Sorting (FACS), and immunomagnetic isolation (MACS), can be used to enrich satellite cell

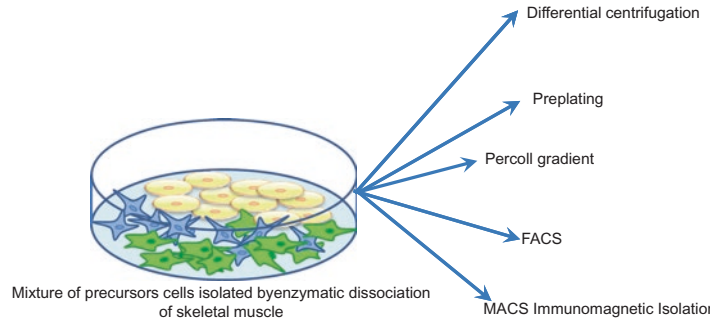

Fig. 1 Scheme of different techniques for the enrichment of satellite cell fraction. Cells prepared by enzymatic digestion of whole muscle tissue are likely to contain a heterogeneous population of precursor cells. Different methods, such as differential centrifugation, preplating and Percoll gradient, FACS, and immunomagnetic isolation, can be used to enrich satellite cell population (*see* the text for details)

population from enzymatic dissociation of skeletal muscles (Fig. 1). In particular, using relevant molecular markers it is possible to sort and culture the different cell populations by the flow cytometry method.

In contrast, single muscle fiber preparations, in which satellite cells retain their normal anatomical position beneath the basal lamina, give rise to a more homogeneous population of satellite cells, since they are free of interstitial and vascular tissue and can therefore be used to investigate satellite cell behavior in the absence of other myogenic cell types.

3.1 Isolation of Satellite Cells from Isolated Myofibers

This method is based on the protocol outlined in Shefer and Yablonka-Reuveni [3] and adapted by Collins et al. [4].

1. Muscles are dissected and digested for 60 min. in 0.2 % (w/v) collagenase type I in Dulbecco's modified Eagle's medium in a 37 °C water bath.
2. Intact myofibers are suspended in 8 mL of plating medium, consisting of DMEM supplemented with 10 % horse serum (Sigma), 0.5 % chick extract, 4 mM L-glutamine, and 1 % penicillin and streptomycin solution. Myofibers are triturated for 5 min with a 19 G needle mounted on a 1 mL syringe. The suspension is passed through a 40 μm cell sieve to remove the myofibers. The remaining satellite cell suspension is centrifuged for 15 min at 450 RCF, and the resultant pellet is resuspended in growth medium [3]. Isolated satellite cells are incubated in growth medium at 37 °C, 5 % CO_2 in a humidified tissue culture incubator.

In addition, detailed methods for the isolation of viable muscle fibers and for grafting of muscle fibers and their associated satellite cells into mouse muscles to assess the contribution of satellite cells to muscle regeneration have been recently reported by Collins and Zammit [5].

3.2 Isolation of Satellite Cells by Enzymatic Dissociation of Skeletal Muscles

Muscle tissue from hind limbs of one mouse will yield enough cells for ten 35 mm or four 6 cm tissue culture dishes.

This method involves three main steps:

1. Dissection (use of enzymes to liberate satellite cells from cleaned and minced muscle).
2. Enrichment of satellite cell fraction.
3. Plating of satellite cells on selected substratum. Alternatively, satellite cells can be cryopreserved prior to plating.

First, it is important to disaggregate the cells from muscle tissue. The extracellular matrix in animal tissues is a complex mixture of collagens and other extracellular matrix proteins. Therefore, a combination of proteolytic enzymes is required for dissociating

tissues. The matrix must be effectively broken down to isolate single cells, without alteration of cellular structures. Pronase and trypsin are largely used for this purpose since both easily destroy components of the basal lamina allowing liberation of satellite cells and other intact cells. However, pronase and trypsin are also destructive to satellite cells and this limits the survival of satellite cells (*see* **Note 1**). An alternative protocol is based on the use of collagenase/dispase, which provides a combination of collagenolytic and proteolytic enzymes required for muscle tissue disaggregation, without alteration of cellular structures and without loss of cell viability.

Second, the initial isolated muscle cells contain a mixture of other cell types, mainly fibroblasts, which grow vigorously in culture and predominate if they are not removed (Fig.1). Different methods can be used to enrich satellite cell population (*see* **Notes 2** and **4**, Fig. 1). The myoblast enrichment protocol takes advantage of the fact that myoblasts adhere to plastic much less avidly than do fibroblasts, and therefore the fibroblasts can be removed from the culture by pre-absorption on plastic tissue culture plates. However, this does not guarantee pure myoblast cell cultures. Addition of β-D-arabinofuranoside (AraC) to the differentiation media can improve the culture of myoblasts since it will select against proliferating cell type (i.e., fibroblasts) and generate pure myotube cultures. AraC (0.3 mg/100 mL) should be added after the first day in differentiation media and left on for a 24 h period.

An alternative method to that described above has been proposed by Montarras et al. [6], who reported a direct isolation of satellite cells for skeletal muscle regeneration. The authors used a *Pax3*$^{GFP/+}$ mouse line to directly isolate Pax3-greenfluorescent protein–expressing muscle satellite cells, by flow cytometry from adult skeletal muscles, as a homogeneous population of small, nongranular, Pax7+, CD34+, CD45−, Sca1− cells. The flow cytometry parameters thus established enabled the authors to isolate homogeneous satellite cells population from transgenic muscles. This technique discloses also the importance of specific animal models to analyze for example the specific effect of homogeneous cell populations on muscle regeneration and repair.

Finally in the MACS immunomagnetic isolation technique, nontarget cells are directly magnetically labeled with a cocktail (CD31/CD45/CD11b/Sca1) of monoclonal antibodies conjugated with MACS MicroBeads, 50-nm superparamagnetic particles. Due to the small size, they do not activate cells and will not saturate cell surface epitopes. The sample is applied to a column placed in a MACS Separator. The unlabeled cells (satellite cells) pass through while the magnetically labeled cells are retained within the column (CD31/CD45/CD11b/Sca1 positive cells). The flow-through can be collected as the Satellite cells pure fraction. Additional positive selection step based on integrin-alpha-7 expression to further increase the purity of satellite cells can be performed.

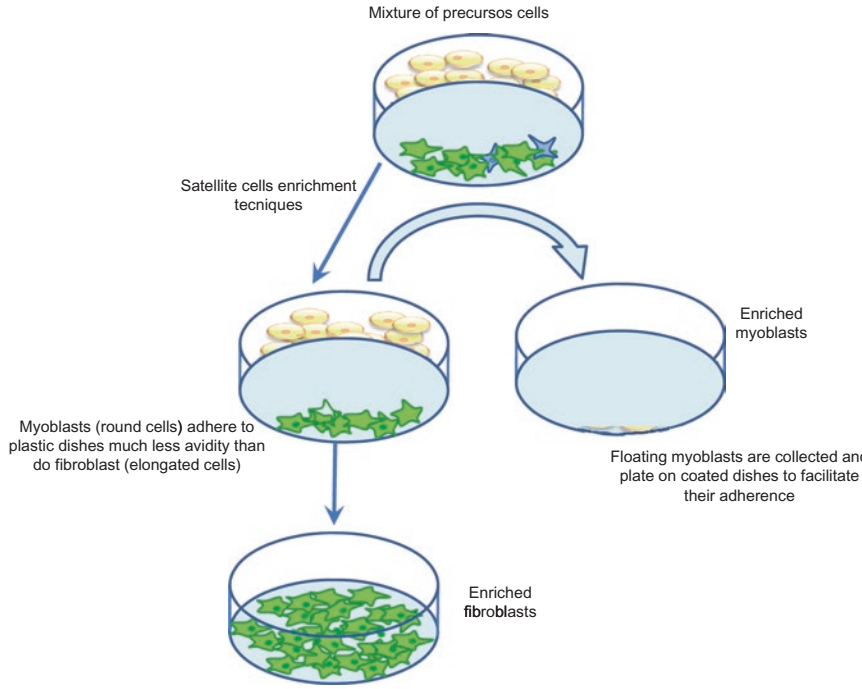

Fig. 2 Schematic representation of satellite cells enrichment by preplating technique

Third, the very characteristics that allow murine myoblast enrichment also require that they ultimately grow on plates coated with specific substrates to facilitate their adherence (Fig.2). Different substrates can be used for this purpose (*see* **Note 3**). Matrigel has been used to facilitate myoblast adhesion on plastic dishes. However, cells attached to Matrigel are hard to release. Fibronectin and laminin are more suitable substrates than Matrigel since they are components of the basal lamina. Collagen represents an alternative substratum and it is commonly used in studies on satellite cell proliferation and differentiation. Once these precautions are taken, primary myoblasts are relatively easy to propagate and spontaneously recapitulate the myogenic differentiation process upon reaching confluence or after sufficient time in culture (Figs.3, 4).

Muscle cells can be cultured in DMEM supplemented with horse or foetal bovine serum and chick embryo extract (CEE). Indeed, mouse primary cultures will grow best with CEE included in the plating media. Mouse myoblasts also experience a long lag period before attaching to the culture plate. For this reason, it is important not to change the media for the first 2 days to allow the cells time to adhere to the substrate.

Isolated satellite cells can also be cryopreserved. For this purpose, satellite cells can be resuspended in DMEM supplemented with 20 % Horse Serum and 10 % DMSO and aliquoted in

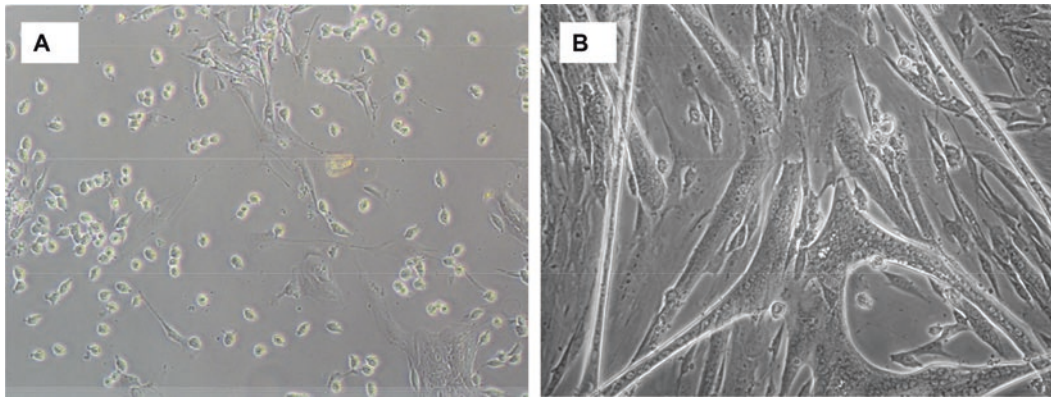

Fig. 3 Morphological analysis of (**a**) proliferating satellite cells, (**b**) terminally differentiated myotubes

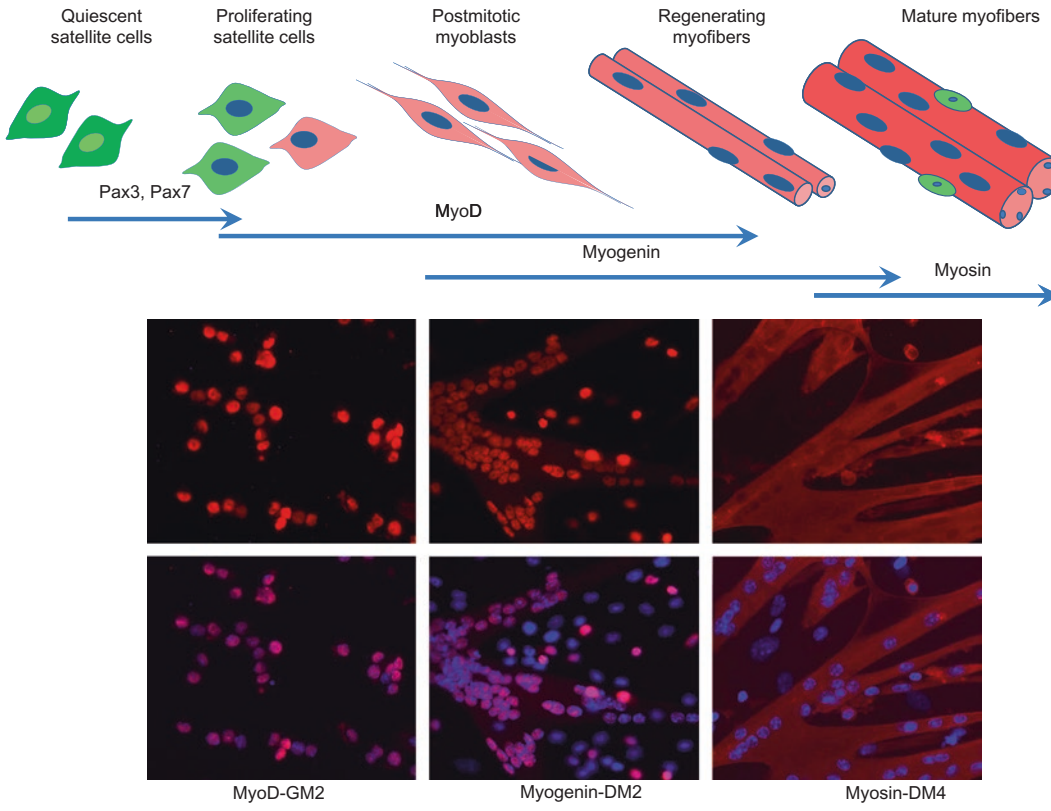

Fig. 4 The molecular signature of satellite cells during differentiation. *Top Panel*: Schematic model outlining the stages of satellite cells activity and the relevant markers expressed by satellite cells during myogenic differentiation. *Bottom panels*: Immunofluorescence analysis of MyoD (after 2 days in GM), Myogenin (after 2 days in DM), and Myosin expression (after 4 days in DM). MyoD identifies proliferating satellite cells, Myogenin identifies committed satellite cells, and Myosin identifies differentiated satellite cells. Nuclei were visualized by Hoechst dye (*blue*). (GM = Growth Medium; DM = Differentiation Medium)

cryovials at a density of 1.0×10^6/vial. Aliquoted satellite cells are placed at −80 °C overnight and then transferred to liquid nitrogen for long-term storage. When required for experimental use, satellite cells are thawed in a 37 °C water bath, centrifuged at 1.200 RCF for 5 min and the cell pellet resuspended in Growth Medium. The differentiation stage can be monitored analyzing MyoD and myosin expression by immunofluorescence analysis.

3.3 Procedure for Isolation of Satellite Cells by Enzymatic Dissociation of Skeletal Muscles

Dissection of muscle hind limbs from adult mice (2–3 month old mice).

3.3.1 Classical Protocol

1. Prepare plates (6 cm Petri dishes) for myoblast culture the day before dissection. Dissolve 10 mg Collagen in 10 mL of 20 % Acetic Acid. Cover the surface of each 6 cm dish with 2 mL of Collagen solution (1 mg/mL). Aspirate the collagen solution off the plates. Place the plates at 37 °C and leave for at least 3 h to dry. Alternatively, take the lid of the plates off and leave the plates under the tissue culture hood to dry.
2. Sacrifice a mouse and place it in a beaker containing 95 % ethanol for 5 min. Subsequently, the mouse is placed in a sterilized beaker containing HBSS or PBS for a minimum of 10 min to remove ethanol and wash the tissues. Dissecting tools should be laid on a paper towel and constantly rinsed with 95 % ethanol. Allow instruments to air dry before touching tissue.
3. Deskin the legs and remove hind and fore limbs. Collect the legs in a petri dish containing HBSS or PBS.
4. Remove any visible fat deposits with forceps; remove bones by using tweezers and scissors to pull muscle tissue away from bone.
5. Place the isolated muscle tissue in a new Petri dish containing HBSS or PBS. All subsequent manipulations are carried out in a tissue culture hood.
6. Remove HBSS or PBS with the Pasteur pipette. Mince tissue with small surgical scissors.
7. Add 10–15 mL/limb of 0.1 mg/mL collagenase type II and transfer minced tissue to a 50 mL conical tube and incubate at 37 °C for 30 min on a rocker. This step is useful to dissociate muscle fibers and to dissolve connective tissue. Centrifuge for 3 min. at 400–500 rpm; remove supernatant and resuspend the pellet in 10–15 mL of 1 mg/mL Collagenase/Dispase and incubate in water bath at 37 °C for 30 min on rocker (Collagenase/Dispase, ROCHE, provides a combination of

collagenolytic and proteolytic enzymes required for muscle tissue disaggregation).

8. Triturate, by pipetting several times, every 10 min. The tissue should be dispersed with no visible clumps.
9. Add an equal volume of plating media and pipette several times.
10. Filter the homogenate using in sequence: 100 μm nylon mesh cell strainer (Falcon 2360), 70 μm nylon mesh cell strainer (Falcon 2340), and 40 μm nylon mesh cell strainer (Falcon 2350) (use fresh strainer as necessary).
11. Collect flow through in a 50 mL comical tube.
12. Centrifuge in a table top centrifuge at 1200 rpm for 10 min. Aspirate supernatant and resuspend the pellet gently in 10 mL of plating media (Growth Medium).
13. Pour the solution into a 100 mm Petri dish and preplate in an incubator (5 % CO_2, 37 °C) for 1 h to remove fibroblasts.
14. Carefully remove the solution from the Petri dish by tilting it to one side. This solution contains the enriched myoblast population.
15. Plate the enriched myoblasts solution in tissue culture dishes coated with collagen.
16. After 48 h change the medium to fresh Growth Medium. It is important not to change the media for the first 2 days to allow the cells time to adhere to the substrate.
17. At about 3–5 days (the myoblasts should present a fusiform phenotype) shift the culture myoblasts from Growth Medium to Differentiation Medium and analyze for MyoD, myogenin, and myosin expression (Fig.4).

3.3.2 Automated Dissociation Protocol

Recently, it has been introduced an automated commercial system that allows the combination of both mechanical and enzymatic treatment for efficient dissociation skeletal muscle (Fig. 5) (*see* **Note 5**). The system utilizes a commercial mix (Miltenyi) of three different enzymes (trypsin-free) for the dissociation while preserving the four non-satellite-cell markers CD31, CD45, CD11b, and Sca1. Once having obtained the hind and fore limbs as indicated in the previous section, it is necessary to follow the following steps;

1. Prepare enzyme mix as indicated in the supplier data sheet.
2. Cut skeletal muscle tissue into small pieces of 2–4 mm.
3. The sample and the enzymes mix are inserted on the Gentle MACS Dissociator and a dedicated protocol (1.5 h) should be performed (37C_SMDK_2).
4. Perform a short centrifugation step to collect the sample material at the tube bottom.

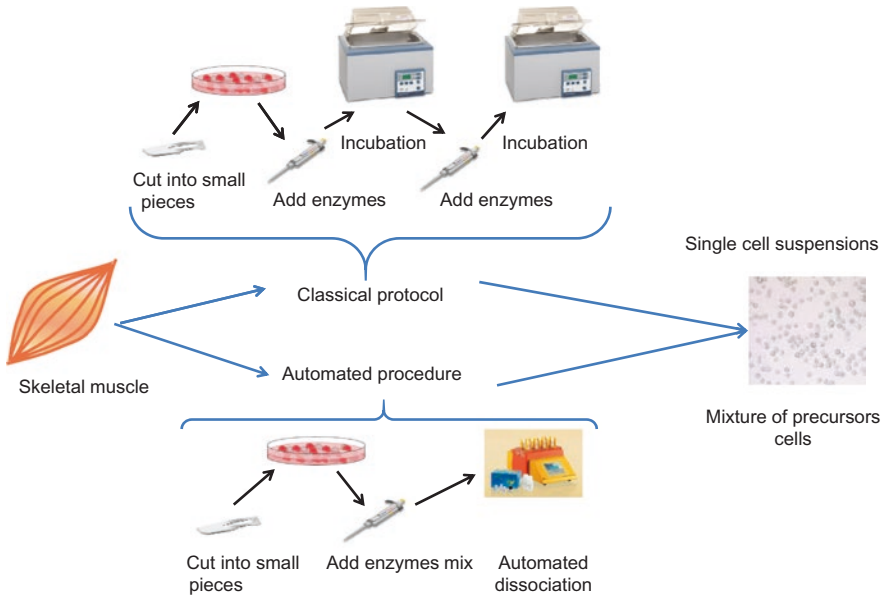

Fig. 5 Scheme of the procedure for isolation of satellite cells by enzymatic dissociation of skeletal muscles. Classical protocol vs. automated procedure

5. Resuspend sample and apply the cell suspension to a strainer, 70 μm, placed on a 15 mL tube.
6. Discard strainer, 70 μm, and centrifuge cell suspension at 300 × g for 20 min. Aspirate supernatant completely.
7. Resuspend cells with an appropriate buffer to the required volume for further applications, for example, resuspend cells in PEB buffer for magnetic cell separation or flow cytometry.

4 Storage of Satellite Cells

In the case of skeletal muscle primary cultures it is better to work with freshly isolated satellite cells, moreover, if necessary, it is possible to cryopreserve these cells.

According to our experience the behavior of cryopreserved satellite cells is not compromised until the third-fourth passage in culture.

4.1 Procedure of Cryopreservation

At 70 % confluence, satellite cells could be collected for the cryopreservation procedure. It is important to monitor the rate of confluence in vitro since if cells are too confluent they will stop to proliferate and begin to spontaneously differentiate to mature myotubes.

1. Detach cells from the Petri dish by the addition of 1.5 mL of 0.25 % trypsin-EDTA and subsequently incubate at 37 °C, 5 % CO_2 for 3–5 min. It is essential to monitor the cell detachment

under an inverted microscope to make sure the cell harvesting is successful by looking at the number of cells left behind. There should be less than 5 %.

2. Rapidly inactivate the trypsin by adding growth medium.
3. Centrifuge (5 min at 0.4 × *g*) and resuspend the cell pellet in filter sterilized freezing medium. Freezing medium contains foetal bovine serum with 10 % dimethyl sulfoxide.
4. Maintain cells at 4 °C for 30 min and then put at −80 °C overnight, before finally being stored in liquid nitrogen until required.

4.2 Thawing Procedure

1. Immerse the cryotube in a water bath heated to 37 °C.
2. When partially thawed, remove the sample should from the water bath and rapidly combine with approximately 1–2 mL of growth medium to dilute the dimethyl sulfoxide.
3. Gently pipette the solution to facilitate thawing.
4. Once fully thawed, combine the cell solution with 9 mL of growth medium and centrifuge at 0.4 × *g* for 5 min to pellet the cellular fraction.
5. Discard the supernatant to remove the dimethyl sulfoxide and gently resuspend the pellet in growth medium.

As mentioned above, we recommend using these cells until passage 3 or 4 to avoid senescence.

5 Notes

1. The temperature and length of exposure of the minced muscle to the pronase and trypsin enzymes should be monitored during the isolation procedure.
2. Preplating is the most commonly used technique for removing fibroblasts.
3. Different substratum can be used to facilitate the adhesion of satellite cells to the Petri dishes, after a careful consideration of pros and cons of each of them:

   Matrigel (cells attached to matrigel are hard to release for counting purpose), Fibronectin (it is a component of basal lamina; expensive), Laminin (more indicates in study of SC differentiation; very expensive), Collagen (commonly used in studies on SC proliferation and differentiation).
4. 20′ on ice can induce the preferential release of satellites cells from the bottom of the Petri dish.
5. The automated protocol presents some advantages if compared to the classical manual isolation protocol: the automated procedure allows a standardization of the method, in addition it can induce less stress on isolated cells.

These aspects are very important in the case of primary cultures obtained from pathological models in which cells could present phenotypic and functional alterations.

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Chapter 13

Isolation of Stromal Stem Cells from Adipose Tissue

Maria Prat, Francesca Oltolina, Silvia Antonini, and Andrea Zamperone

Abstract

Adipose tissue has been shown to be particularly advantageous as source of mesenchymal stem cells (MSCs), because of its easy accessibility, and the possibility of obtaining stem cells in high yields. MSCs are obtained from the so-called Stromal Vascular Fraction, (SVF), exploiting their property of adhering to plastic surfaces and can be further purified by positive or negative immunomagnetic selection with appropriately chosen antibodies. These cells (Stromal Stem Cells, SSCs) can then be directly analyzed, frozen in liquid nitrogen, or expanded for further applications, e.g., for tissue engineering and regenerative medicine. The methodology described here in detail for SSCs isolated from mouse subcutaneous adipose tissue can be applied to human tissues, such as epicardium.

Key words Adipose tissue, Mesenchymal stem cells, Stromal stem cells, Cell isolation, Cell culturing, Cell differentiation, Immunophenotyping

1 Introduction

Mesenchymal Stem Cells (MSCs) are among the most promising cells for therapeutic potential in the field of regenerative medicine [1]. Among the different tissues of mesodermal origin, which contain them, adipose tissue has been reported to harbor them since 2001 [2]. The advantages of this tissue as source of adult MSCs, when compared with the more conventional and traditional bone marrow, whose properties are similar, are the followings: it can be harvested easily and repeatedly in higher amounts, with higher yields of stem cells [3], and with lower donor discomfort and risk of morbidity. Stem cells can be easily enriched by simple plating on a plastic surface of the stromal vascular fraction (SVF) obtained from the adipose tissue [4] and can be further purified on the basis of stemness cell surface markers, by immunomagnetic cell (IMAC) sorting. Adipose tissue can be recovered from distinct sites, such as subcutaneous, omental, and epicardium [5]. While cells (herein called SSCs for stromal stem cells) display basic similar properties, the question is open whether the niche from which they derive may somehow influence their destiny, since they could have received

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some imprinting from the microenvironment [6]. As an example, it seems that SSCs from epicardium may be a better source for progenitors of cardiomyocytes [7]. Since a protocol for the derivation of primary human stem cells from epicardium was recently published [7], the present paper describes namely the isolation of SSCs from subcutaneous adipose tissue of the mouse. The same method was found to be applicable also to human adipose tissue obtained from epicardium as well as from subcutaneous fat, obtained upon tissue resection. It is worth mentioning here that no significant differences were observed when SCCs were prepared from human subcutaneous adipose tissue recovered through liposuction [8, 9]. Finally, the characterization, as immunophenotype and multilineage potentials, of the isolated SSCs is also reported.

2 Materials

This protocol describes the isolation of adult mesenchymal stem cells from s.c. and epididymal fat pads of 11-week-old mice and the subsequent establishment of explant cultures. The same strategy with small modifications can be applied to obtain human stromal stem cells (SSCs) from subcutaneous adipose tissue and epicardium. All tissue culture materials and reagents must be sterile. Tools for isolation should be cleaned thoroughly in 70 % ethanol prior to use. All procedures must be carried out in a laminar flow hood under strict sterile conditions. SSCs are separated from other remaining cells by positive or negative selection using magnetic cell sorting. Once SSC explant cultures have been established it is possible to expand the cell population for its storage in liquid nitrogen, its characterization, and its use in larger experiments.

All the experiments reported herein with animal and human tissue specimens were carried out according to guidelines approved by the European Community Directive for Care and Italian Laws on animal experimentation (Law by Decree 116/92) and the Institutional Review Board (IRB) of Novara (Italy) (Comitato Etico Interaziendale, protocol No. 338/CE, study CARDIOCELL, CE 54/10, approved on June 22, 2010) respectively.

2.1 Materials Required for Isolation of SSC from Adipose Tissue

1. Antibiotics: penicillin-streptomycin mix (100× solution).
2. Dulbecco's Phosphate-buffered saline (PBS) supplemented with antibiotics (100 IU/mL penicillin, and 100 mg/mL streptomycin).
3. Biopsy samples: this protocol utilizes s.c. and epididymal fat pads from mouse, as well as human subcutaneous and epicardial adipose tissue samples excised from patients undergoing cardiac surgery collected in PBS supplemented with antibiotics.

4. 0.1 % gelatin solution: to be prepared as follows: 5 g of porcine gelatin dissolved in 500 mL distilled water and autoclaved. It can be stored up to 6 months at 4 °C. For use, dilute the gelatin to a 0.1 % solution in PBS. It can be stored up to 3 months at 4 °C.
5. Fetal Bovine Serum (FBS): Heat-inactivate at 55 °C for 30 min.

Aliquot and store frozen. Thaw at 4 °C.

6. SSC culture medium: Ham's F-12K (Kaighn's) Medium. Claycomb medium 2:1, supplemented with 2 mM L-glutamine, 10 % fetal bovine serum, 100 IU/mL penicillin, and 100 mg/mL streptomycin, warmed (37 °C) (from now on called complete medium).
7. Collagenase A type I.
8. Dulbecco's Minimum Essential Medium (DMEM).
9. Red blood cell lysis buffer: 2.06 g/L Tris–HCl Base, pH 7.2, 7.49 g/L NH4Cl. Sterile filter after preparation. Can be kept at room temperature for 4 weeks.
10. Falcon 40 μm cell strainers for mouse cells. Falcon 100 μm cell strainers in the case of human cells.
11. 6-well Corning Costar cell culture plates.
12. anti-Sca-1 Microbeads kit (FITC), mouse, FITC- anti-CD31, and FITC-anti CD45 antibodies.
13. MACS anti-FITC Microbeads.
14. Column buffer: phosphate buffered saline (PBS; pH7.2), 0.5 % FBS, 2 mM EDTA.
15. MACS LD columns.
16. MiniMACS and MidiMACS separation unit.
17. 50 and 15 mL plastic conical tubes.
18. 25, 10, and 1 mL disposable plastic pipettes.
19. A swing-out centrifuge with buckets for 50 and 15 mL tubes.
20. Cell incubator set at 100 % humidity, 37 °C, and 5 % CO_2 in air.
21. 100 × 25-mm^2 petri dishes for dissection.
22. Two sets of forceps (approximately 0.5-mm tip size).
23. Swann Morton No. 21 disposable scalpels, sterile.

2.2 Materials Required for the Expansion, Culturing, and Freezing of SSCs

1. 0.1 % gelatin solution.
2. Dulbecco's Phosphate-buffered saline (PBS).
3. SSC cultures (*see* Protocol 3.6).
4. SSC culture complete medium: Ham F12: Claycomb medium 2:1, supplemented with 2 mM L-glutamine, 10 % fetal bovine

serum, 100 IU/mL penicillin, and 100 mg/mL streptomycin, warmed (37 °C).

5. 0.05 % Trypsin-EDTA solution warmed (37 °C).
6. Appropriate-sized tissue culture plates or flasks.
7. Freezing medium: 10 % Dimethyl Sulfoxide (DMSO)- 90 % fetal bovine serum.
8. Cryotubes.
9. Cell incubator set at 100 % humidity, 37 °C and 5 % CO_2 in air.
10. Mr. Frosty™ Freezing Container, containing Isopropyl alcohol.

2.3 Immunophenotyping of Cells

2.3.1 Analysis at Fluorescence Microscope

1. 0.1 % gelatin solution (*see* recipe).
2. Round 13-mm sterile glass coverslips.
3. 24-well Corning Costar cell culture plates (15.9-mm diameter).
4. Dulbecco's Phosphate-buffered saline (PBS).
5. 4 % paraformaldehyde solution: 4 % PFA in PBS.
6. Permeabilization-blocking solution: 0.1 % Triton X-100, 1 % bovine serum albumin (BSA), 5 % goat serum in PBS.
7. Solution for primary antibody dilution and first washings: PBS-TX-BSA: 0.1 % Triton X-100, 1 % bovine serum albumin (BSA) in PBS.
8. Solution for second and third washings: PBS-TX: 0.1 % Triton X-100 in PBS.
9. Primary antibodies (*see* Table 1).
10. Appropriate FITC-conjugated secondary antibodies (anti-Mouse IgG (whole molecule)–FITC antibody produced in goat; anti-Mouse IgG F(ab′)2-Rhodamine antibody produced in goat.
11. DAPI (5 μg/mL in PBS).
12. SlowFade®: Gold antifade reagent (Invitrogen).
13. Clear nail polish.
14. Superfrost Plus microscope slides.
15. Parafilm.
16. Light-proof microscope slide box.
17. Forceps for glass coverslips.
18. Fluorescence microscope.

2.3.2 Analysis at Flow Cytometry

Most of the reagents (namely the antibodies) are the same as for the analysis at fluorescence microscopy.

1. EDTA solution (5 mM EDTA in PBS).
2. 1.5 mL Eppendorf tubes.

Table 1
Antibodies used for cell isolation and analysis

Antigen	Ab supplier
FITC-mouse-Sca-1	MiltenyiBiotec
FITC-human-CD31	Biolegend
FITC-human-CD45	Biolegend
PE-mouse-Sca-1	BD Pharmingen
Mouse-CD90	Immunotools
PE-mouse-CD34	Biolegend
FITC-mouse-CD44	Biolegend
FITC-mouse-CD106	Biolegend
FITC-mouse-CD31	Biolegend
Human-CD44	Biolegend
Human-CD90	Biolegend
Human-CD105	Biolegend
c-kit/CD117	Biolegend
human-CD34	Biolegend

3. Polystyrene tubes for cytometry (BD Falcon).
4. Cytofluorometer.

2.4 Materials Required for the Evaluation of Multipotency of SSC from Adipose Tissue

1. Medium for adipogenic differentiation: DMEM:F12 containing 10 % FBS, 0.5 μM 1-methyl-3 isobutylxanthine, 1 μM dexamethasone, 10 μg/mL insulin, and 100 μM indomethacin.
2. Medium for osteogenic differentiation: DMEM:F12 containing 10 % FBS, 100 nM dexamethasone, 10 mM β-glycerophosphate, and 0.05 mM L-SSCorbic acid-2-phosphate.
3. Medium for chondrogenic differentiation: hMSC Chondro BulletKit (Lonza) containing TGF Beta 3 for HMSC Chondro diff (Lonza).
4. Dulbecco's Phosphate-buffered saline (PBS).
5. 4 % PAF solution.
6. Killik.
7. Oil-Red O.
8. 40 mM solution of Alizarin Red S.
9. 1 % Alcian blue in 3 % acetic acid, pH 2.5.
10. Superfrost Plus microscope slides.
11. Microtome.

3 Methods

3.1 Sample Preparation

1. Place the adipose tissue sample into a sterile 100 × 25-mm^2 petri dish and rinse with 5 mL PBS supplemented with antibiotics.
2. Using a scalpel cut the sample in pieces of 1–3 mm (*see* **Note 1**).

3.2 Collagenase Digestion

1. Make up collagenase solution just prior to digestion. The final volume required is half that of the washed adipose tissue volume. Add powdered collagenase to DMEM at a final concentration of 0.2 %. We dissolve the required amount of collagenase into 40 mL of DMEM, then filter sterilize into the remaining working volume. Add antibiotics.
2. Transfer the washed adipose tissue to a 50 mL Falcon tube.
3. Add collagenase solution.
4. Resuspend the adipose tissue fragments by shaking the flasks vigorously for 5–10 s.
5. Incubate at 37 °C on a shaker for 30 min–1 h, manually shaking the tube vigorously for 5–10 s every 15 min.
6. On completion of the digestion period, the digested adipose tissue should have a "soup like" consistency.
7. Add FBS to a final concentration of 10 % to stop collagenase activity.

3.3 Separation of the Stromal Vascular Fraction

1. Centrifuge at room temperature at 300 × *g* for 10 min at room temperature.
2. After centrifugation, use a 50 mL pipette to aspirate the floating adipocytes, lipids, and the digestion medium. Leave the SVF pellet in the tube.

3.4 Removal of Red Blood Cells

1. Resuspend thoroughly SVF pellet in 20 mL of cell lysis buffer at room temperature.
2. Incubate at room temperature for 10 min.
3. Centrifuge at 300 × *g* for 10 min and aspirate the cell lysis buffer and wash twice.

3.5 Filtration

1. Resuspend SVF pellets thoroughly in 2 mL of washing medium using a 1 mL pipette.
2. Pipet the cells up and down several times to reduce clumping.
3. Pool the pellets into a 15 or 50 mL tube.
4. Allow undigested tissue clumps to settle by gravity for ~1 min.
5. Aspirate and pass the suspended cells through 40 μm cell strainers. (100 μm cell strainers if SVF from human tissues).

3.6 Plating and Culture of Cells from SVF

1. Coat plates with gelatin as follows:
   Pipet ample volume of 0.1 % gelatin solution in PBS to cover the surface of the culture plate or flask. Allow to stand for 15 min at 37 °C and then aspirate the remaining gelatin solution (*see* **Note 2**).
2. Seed the filtrate in the gelatin-coated tissue culture plates. Moreover, seed the small fragments recovered from digestion: four to five pieces of tissue per well (6-well plate; 34.8-mm well diameter) should be sufficient. In the latter case, add a minimum amount of medium, so that the sample does not get dry.
3. After the cells and digested fragments have all been plated, place the plate into a humidified, 37 °C, 5 % CO_2 incubator for 2 h. This allows the fragments to adhere to the plate.
4. Check that the pieces are not floating. If they are, they can be pushed back down gently with forceps.
5. After 4 h, add further 100 μL to each fragment and place back to the incubator.
6. Next day remove the dish from the incubator and gently add 1 mL SSC culture medium to each well and then place back into the incubator. Cells should begin to grow after about 72 h, but can take up to 5 days. At the same time, some cells start to migrate from the fragments and to grow.
7. Once the cells have started to grow, the medium can be replaced every 3–4 days (*see* **Notes 3** and **4**).

3.7 Immunoselection of Stromal Stem Cells

Stromal stem cells are separated from remaining cells using immunomagnetic cell (IMAC) sorting. Depending on the origin of the cells a different strategy can be used. (a) In the case of mouse cells, stromal stem cells can be selected positively for their expression of the Sca-1 marker. (b) In the case of human cells, stromal stem cells are generally selected negatively, after the removal of unwanted contaminating $CD31^+$ endothelial cells and $CD45^+$ leukocytes CD45. In some cases this step can precede step Subheading 3.6.

3.7.1 Positive Selection of Mouse Sca-1$^+$Stromal Stem Cells

1. Detach cells by the 5 mM EDTA solution, transfer cells to a 15 mL tube, and centrifuge at 300 × *g* for 10 min at 4 °C using a low brake.
2. Resuspend the cell pellet in column buffer and label with anti-Sca-1-FITC-conjugated antibodies according to the manufacturer's recommendations. We resuspend cells in 100 μL of column buffer and add 10 μL of each antibody per 10^7 cells.
3. Mix well and incubate for 15 min in the dark at 4 °C (resuspend the cells after 7 min of incubation).

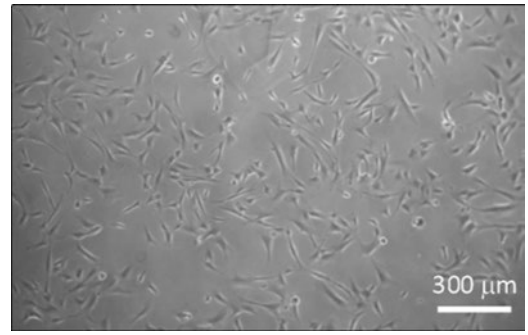

Fig. 1 Mouse stromal stem cells (SSCs) purified from collagenase-digested and selected for the expression of Sca-1. The cells have a spindle fibroblast-like morphology

4. Wash the cells to remove unbound antibody by adding 2 mL of column buffer per 10^7 cells. Centrifuge at 300 × *g* for 10 min at 4 °C using a low brake setting.
5. Aspirate the supernatant completely and resuspend the cell pellet in 90 µL of column buffer per 10^7 cells. Add 10 µL of MACS anti-FITC magnetic microbeads per 10^7 cells.
6. Mix well and incubate for 15 min at 4 °C (resuspend the cells after 7 min of incubation).
7. Wash the cells to remove unbound beads by adding 2 mL of column buffer per 10^7 cells. Centrifuge at 300 × *g* for 10 min at 4 °C using a low brake setting.
8. Aspirate the supernatant completely and resuspend the cell pellet in 500 µL of column buffer.
9. Place a MACS LD column onto the MiniMACS (for up to 10^7 cells) or MidiMACS (for >10^7 cells) separation unit or onto a compatible unit.
10. Prepare the column by washing with 2 mL of column buffer.
11. Apply the cell suspension to the column and collect the flow-through cells in a 15 mL tube.
12. Wash unlabeled cells through the column by twice adding 1 mL of column buffer.
13. Remove the column from the magnetic field, elute the magnetically retained cells by applying the piston, and wash cells with PBS.
14. Seed cells in a culture cell plate at a density of 5–10 × 10^3/cm^2. Once attached cells display a spindle fibroblast-like morphology (Fig. 1).

3.7.2 Negative Selection of Human Stromal Stem Cells

1. Detach cells by the 5 mM EDTA solution, transfer cells to a 15 mL tube, and centrifuge at 300 × *g* for 10 min at 4 °C using a low brake.

2. Resuspend the cell pellet in column buffer and label with anti-CD31 FITC-conjugated and anti-CD45 FITC-conjugated antibodies according to the manufacturer's recommendations. We resuspend cells in 100 μL of column buffer and add 10 μL of each antibody per 10^7 cells.
3. Mix well and incubate for 15 min in the dark at 4 °C (resuspend the cells after 7 min of incubation).
4. Wash the cells to remove unbound antibody by adding 2 mL of column buffer per 10^7 cells. Centrifuge at 300 × *g* for 10 min at 4 °C using a low brake setting.
5. Aspirate the supernatant completely and resuspend the cell pellet in 90 μL of column buffer per10^7 cells. Add 10 μL of MACS anti-FITC magnetic microbeads per 10^7 cells.
6. Mix well and incubate for 15 min at 4 °C (resuspend the cells after 7 min of incubation).
7. Wash the cells to remove unbound beads by adding 2 mL of column buffer per 10^7 cells. Centrifuge at 300 × *g* for 10 min at 4 °C using a low brake setting.
8. Aspirate the supernatant completely and resuspend the cell pellet in 500 μL of column buffer.
9. Place a MACS LD column onto the MiniMACS (for up to 10^7 cells) or MidiMACS (for $>10^7$ cells) separation unit or onto a compatible unit.
10. Prepare the column by washing with 2 mL of column buffer.
11. Apply the cell suspension to the column and collect the flow-through cells in a 15 mL tube.
12. Wash unlabeled cells through the column by twice adding 1 mL of column buffer.
13. Collect the total effluent and perform a cell count.

3.8 Culture, Passaging, and Freezing of Stromal Stem Cells

1. Coat plates with gelatin as in **step 1**, Subheading 3.6.
2. Aspirate the medium from the ASC cultures and gently wash the cells twice, each time with 2 mL PBS. Rather than aspirating the medium, it can be pipetted off and transferred into a sterile. 15-mL Falcon tube. This "conditioned" medium can be stored up to 6 months at −20 °C and used to supplement fresh SSC culture medium later (it may contains growth factors useful for cell proliferation).
3. Add 0.05 % Trypsin/EDTA and incubate for a maximum of 5 min at 37 °C to detach cells. A total of 200 μL trypsin solution is sufficient to detach cells from one full well of a 6-well plate (34.8-mm diameter).
4. Wash the cells off the plate using 2 mL, fresh SSC culture medium. If required, dilute the cells to allow the appropriate

split ratio with more SSC complete medium. Transfer cell suspension to the prepared culture dish. The cells can be resuspended in a 1:1 mixture of fresh SSC culture medium and the previously collected "conditioned" medium to help prevent cell death. Cells should adhere to new culture dish as long at the final trypsin concentration is lower than 10 %.
To remove trypsin from the cultures, detached cells can be resuspended in warm medium, transferred into a 15-ml Falcon tube, and centrifuged for 5 min at 300 × *g*, 20–37 °C. The medium can then be aspirated and the pellet resuspended in fresh SSC medium for plating (*see* **Notes 5–7**).

5. Alternatively, to freeze cells [10], resuspend the pellet in the iced freezing medium (*see* **Note 8**) and put the cryotubes in precooled Mr. Frosty™ Freezing Container and store it at −80 °C. After 3 days, store the cyotubes in a liquid nitrogen tank.

3.9 Characterization of SSC

The isolated and cultured cells must be analyzed for their properties to establish and confirm their identity as stem cells. This can be carried out by analyzing their immunophenotype and stemness potential. The immunophenotype can be analyzed by fluorescence microscopy or by the more accurate flow cytometry, which, however, requires a higher number of cells. All the incubations in which fluorescent reagent is used must be carried out in the dark, and samples should be exposed the minimum necessary to light—only the level necessary to work—while manipulating them.

3.9.1 Experiments for Fluorescent Microscopy Observation

1. Plate SCCs on glass coverslip, coated with 0.1 % gelatin, as described at **step 1**, Subheading 3.6, placed in 24-well plates. Let them reach 50–70 % confluence.
2. Aspirate medium from wells.
3. Fix with 4 % PAF solution at room temperature for 15 min.
4. Wash two times with PBS, and aspirate the last washing.
5. Add the permeabilization/blocking solution and incubate at room temperature for 2 h or at 4 °C O/N.
6. Add the primary antibody, at the appropriate dilution in 200 μL, and incubate at room temperature for 60–90 min or at 4 °C O/N on a rocking platform, in a moist chamber, taking care that cells are maintained wet. Alternatively, put a drop of 20 μL containing the appropriate dilution of the antibody on Parafilm and transfer upside-down (cells must be in contact with the drop) the glass (*see* **Note 8**).
7. Wash once with the washing solution PBS-TX for 10 min (glass coverslip are returned to 24-well plates, in case incubation with primary antibody was carried out on parafilm).
8. Wash twice with PBS for 10 min and aspirate the last washing.

9. Incubate with the appropriate fluorescent secondary antibody and DAPI in PBS at room temperature for 45 min, as described at Subheading 3.9, **step 6**.
10. **Steps 10–11**as described at **steps 7–8**.
11. Mount the coverslips, cell side down, onto the microscope slides using 5 μL of Slowfade. Remove excess liquid with tissue and seal the edges with clear nail polish to prevent drying out.
12. Store at 4 °C for at least 8 h.
13. Visualize under the fluorescence microscope (*see* **Note 9**).

3.9.2 Analysis at Flow Cytometry

All steps are as for phenotyping for the fluorescence microscopy observation, except minor modifications and washings that are carried out by centrifugation.

1. Detach cells with the EDTA solution.
2. Transfer them in Eppendorf tubes, and wash twice.
3. Fix with 4 % PAF solution at room temperature for 10 min.
4. Wash three times with PBS, and aspirate the last washing.
5. Add the permeabilization/blocking solution and incubate at room temperature for 45 min.
6. Add the primary antibody, at the appropriate dilution in 100 μL PBS, and incubate at room temperature for 60–90 min, manually shaking the tube vigorously for 5–10 s every 15 min.
7. Wash once with the washing solution PBS-TX for 10 min, and twice with PBS, aspirate the last washing.
8. Incubate with the appropriate secondary antibody for 45 min.
9. Wash twice in PBS for 15 min.
10. Observe samples at the flow cytometer (*see* **Note 10**).
11. Calculate the percentage of cells labeled by the different fluorescent antibodies relative to the total cell number, whose nuclei are stained in blue by DAPI (Fig. 2).

    Cytograms display the cell populations labeled with fluorescent antibodies as compared with the unlabeled untreated control cell populations (Fig. 3).

3.10 Evaluation of Multipotency

By definition stem cells are able to proliferate and differentiate toward different lineages. MSCs can differentiate toward the adipogenic, the osteogenic, and the chondrogenic lineages.

3.10.1 For Adipogenic Differentiation

1. Plate cells onto 35 mm dishes (2×10^4 cells/cm^2) and culture them in adipogenic medium.
2. Change the medium every 4 days, for about 14–20 days, when cells change aspect, acquiring the typical morphology of adipocytes with bright vacuoles.
3. Wash with PBS.

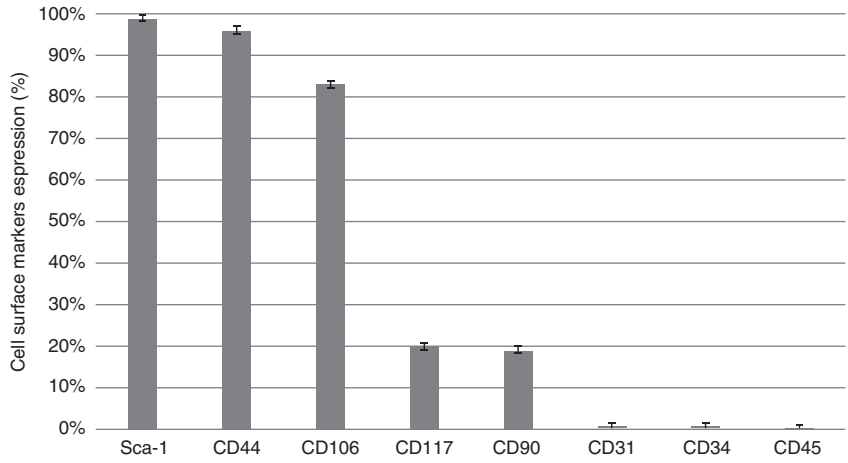

Fig. 2 Flow cytometric analysis carried out on mouse SSCs isolated from ependymal and subcutaneous adipose tissue for different mesenchymal stemness markers (Sca-1, CD44, CD106, CD117, CD90) and control endothelial (CD31) or hemopoietic (CD34, CD45) markers

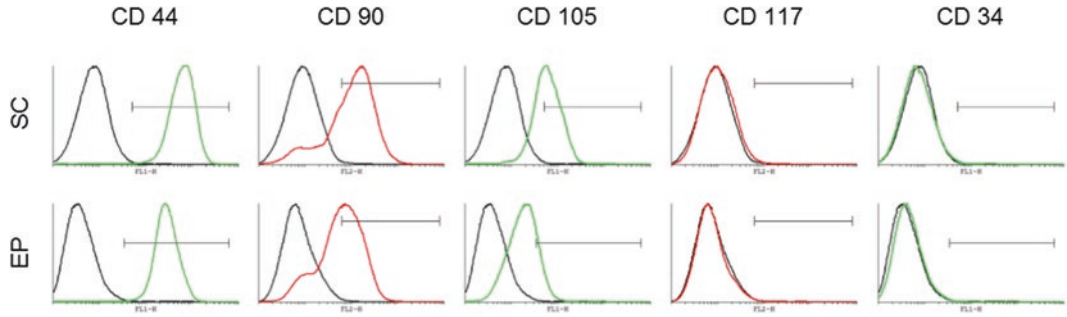

Fig. 3 Representative cytograms of the flow cytofluorometric analysis performed on SSCs isolated from human subcutaneous (SC) and epicardial (EP) adipose tissue. Both cell samples are positive for the mesenchymal stem cell markers CD44, CD90, CD105 and negative for CD117 and CD34

4. Fix with 4 % PAF solution for 10 min at 4 °C.
5. Stain with Oil-Red O.
6. Visualize the presence of lipid vacuoles under a fluorescence microscope.

3.10.2 For Osteogenic Differentiation

1. Plate cells onto 35 mm dishes (2×10^4 cells/cm^2) and culture them in osteogenic medium.
2. Change the medium every 4 days, for about 14–20 days.
3. Wash with PBS.
4. Fix with 4 % PAF solution for 10 min at 4 °C.
5. Stain with alizarin red S solution.
6. Visualize the presence of calcium deposits under a light microscope.

3.10.3 For Chondrogenic Differentiation

1. Culture SSCs as a "pellet" in 15 mL centrifuge tubes in chondrogenic differentiation medium.
2. Continue the culture for 40 days, changing the medium every second day.
3. Wash with PBS.
4. Fix with 4 % PAF solution for 10 min at 4 °C.
5. Include in Killik solution, and freeze at −80 °C.
6. Cut 5 μm sections.
7. Fix again as before and wash with PBS.
8. Stain with Alcian blue solution for 30 min and rinse.
9. Observe under a light microscope.

4 Notes

1. Viable explant cultures are easier to establish from large samples. Tissue pieces of 0.5 × 0.5 cm^2 or larger are recommended.
2. Make sure gelatin solution is completely removed and has had time to dry. If the plates are too wet, the cells will not adhere.
3. The cells grow slowly and do not require frequent medium changes. Refreshing the medium too frequently can hinder cell growth.
4. Rather than replacing the medium entirely, remove half (0.5 mL) of the old medium and top up to 1.0 mL with fresh medium to concentrate cell-secreted mitogenic factors and enhance growth and survival.
5. When splitting SSC cultures, the passage ratio should be no higher than 1:1.
6. Cultures should be 95–100 % confluent before passaging.
7. If SSC begin to die, or show signs of suffering, passaging to a smaller surface area can help recovery and growth.
8. Cells must be in exponential growth, and the day before medium has to be changed. Cells must be resuspended at a concentration of 2–5 × 10^6/ml.
9. Avoid that samples dry, by performing every incubation in a humidified chamber.
10. Samples can be stored at 4 °C in the dark till one week.

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Chapter 14

Human-Induced Pluripotent Stem Cell-Derived Mesenchymal Stem Cells as an Individual-Specific and Renewable Source of Adult Stem Cells

Glen Lester Sequiera, Sekaran Saravanan, and Sanjiv Dhingra

Abstract

This chapter deals with the employment of human-induced pluripotent stem cells (hiPSCs) as a candidate to differentiate into mesenchymal stem cells (MSCs). This would enable to help establish a regular source of human MSCs with the aim of avoiding the problems associated with procuring the MSCs either from different healthy individuals or patients, limited extraction potentials, batch-to-batch variations or from diverse sources such as bone marrow or adipose tissue. The procedures described herein allow for a guided and ensured approach for the regular maintenance of hiPSCs and their subsequent differentiation into MSCs using the prescribed medium. Subsequently, an easy protocol for the successive isolation and purification of the hiPSC-differentiated MSCs is outlined, which is carried out through passaging and can be further sorted through flow cytometry. Further, the maintenance and expansion of the resultant hiPSC-differentiated MSCs using appropriate characterization techniques, i.e., Reverse-transcription PCR and immunostaining is also elaborated. The course of action has been deliberated keeping in mind the awareness and the requisites available to even beginner researchers who mostly have access to regular consumables and medium components found in the general laboratory.

Key words Mesenchymal stem cells, Induced pluripotent stem cells, Differentiation, Regenerative medicine, Non-variable MSC source

1 Introduction

Induced pluripotent stem cells (iPSCs) are embryonic stem cell (ESC)-like cells. They can be established either through somatic cell nuclear transfer or through forced overexpression of a few master transcription factors, famously demonstrated by Dr. Shinya Yamanaka in his Nobel-worthy work [1, 2]. They have the capacity to differentiate into many cell types and have been proven to be capable of long-term culture [3–6]. iPSCs can be sourced by reprogramming any cell types – progenitors, germ cells, or somatic. This allows for a patient-specific iPSC generation, which would pave the way for recipient-tailored screening and therapeutic avenues.

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DOI 10.1007/978-1-4939-6756-8_14,

Former ways of reprogramming through viral-integration vectors were deemed to be not appropriate for the translational research [7]. As of late, new methods of reprogramming, through non-viral vectors, mRNA, protein, and small molecules, have opened up the possibility of safe iPSC generations. Though there are differences between iPSC and ESC, they both share few of the properties namely long-term culture, ability to differentiate over a long period of time, and comparable cellular stability [8, 9].

As iPSC themselves are too risky for transplantation, it is more conducive to differentiate them into either committed progenitors or terminally differentiated cells. One of the lucrative progenitor population is the mesenchymal stem cells (MSCs).

MSCs have been regularly tested and proven to be safe for transplantation. They have not been associated with any adverse effects in the numerous clinical trials going on [10]. MSCs have the unique properties of immune privilege, ability to differentiate into a few cell types and easy sourcing. Further, they have been found to have a strong paracrine effect on recruiting and helping increased proliferation of endogenous resident stem cells/progenitors [11]. On the other hand, the present extraction of MSCs usually involves invasive techniques like bone marrow biopsy or liposuction. Also, the quality of the MSCs deteriorates as the donor ages. The MSCs cannot be cultured indefinitely, and long-term storage of MSCs has been known to be associated with quality reduction. Multiple doses of MSCs seem to be the order of the day to ensure continuing benefits.

iPSC-MSCs are not only person-specific, reducing the possibility of immunorejection, but they are also inexhaustible. In terms of therapeutical considerations, genetic corrections of iPSCs, given their lasting renewability, are a better and assured approach compared to genetic modification of MSCs, which are limited in their self-renewability. An epigenetic switch occurs in iPSC making them embryonic in nature [12]. These changes wipe out the effect of aging, which are then maintained in the iPSC-MSCs[13]. iPSC-MSCs have also been proven to be alike adult tissue-derived MSCs in their structure and morphology as well as their function [14].

Here, we have described a simple method for differentiation of human iPSCs(hiPSC) to MSCs. As clinically approved iPSC generation and establishment become more acceptable and regular, MSC derived from these iPSCs would provide better benefits of person-specific tailoring of cell lines and inexhaustible source. The outlined directions will provide a step-by-step approach for the regular maintenance of hiPSC and their subsequent differentiation into MSCs using specific medium and detailed procedures. The protocol approximately takes 10–12 days to show emergence of MSC (Fig. 1). Procedures for isolation, identification, purification, and general maintenance of these hiPSC-MSC have been elaborated keeping in mind to employ easily accessible medium and reagents.

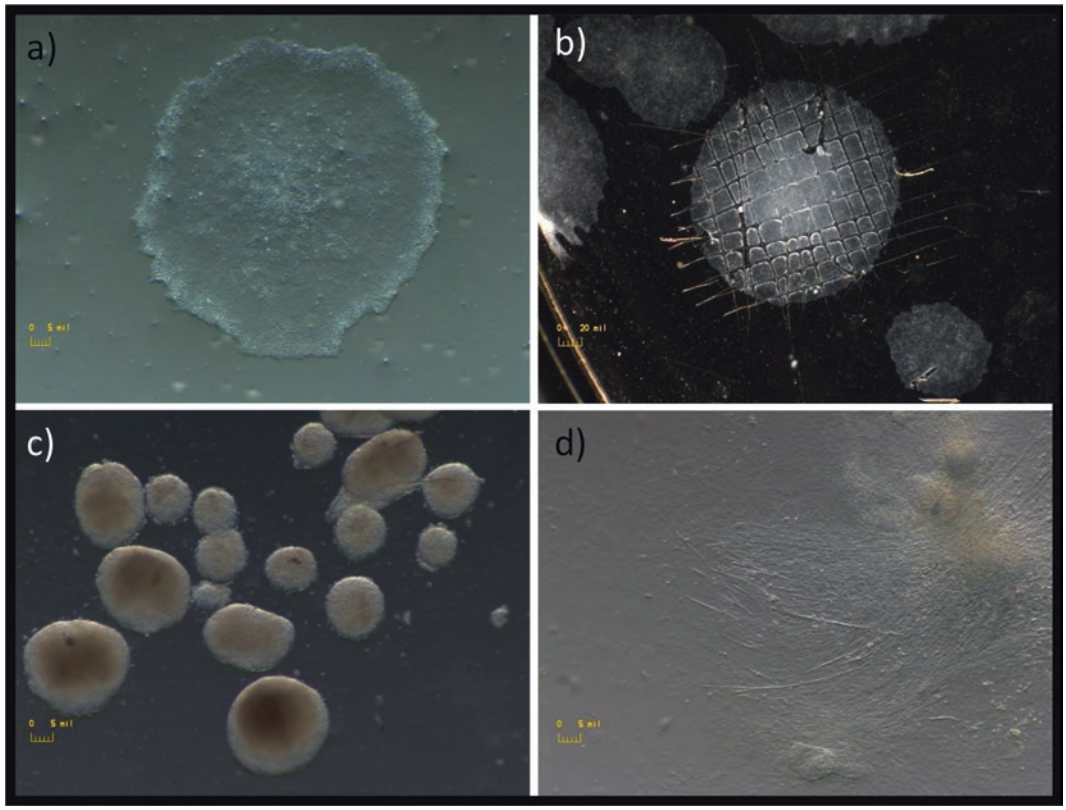

Fig. 1 (**a**) Flat hiPSC colony, (**b**) Manual passaging of hiPSC colony using a needle for gridding, (**c**) Floating embryoid bodies, (**d**) Cells migrating out of embryoid body

2 Material

2.1 Cells

1. Human-induced pluripotent stem cells (in house reprogramming/commercially available).

2.2 Supplies and Equipment

1. 15 mL and 50 mL conical centrifuge tubes.
2. 1.5 mL centrifuge tubes.
3. 6 cm tissue-culture grade plates.
4. Tissue culture flasks.
5. Low attachment 6-well plates.
6. Cell counter and specific slides.
7. Glass slides and cover slips.
8. Pipettes and pipette tips.
9. Sterile waste aspiration system.
10. Sterile needles.
11. Cell lifters.

12. Sterile 10 cm non-tissue culture petridishes.
13. Inverted microscope.
14. Fluorescence microscope.
15. Macroscope.
16. Centrifuge with multiple rotors, buckets, and holders.
17. CO_2 incubators.
18. −80 °C freezers.
19. Liquid nitrogen freezer.

2.3 Reagents and Buffers (See Note 1)

1. Essential 8™ medium (Life Technologies).
2. Geltrex (Life Technologies).
3. Phosphate Buffered Saline (PBS), Calcium/Magnesium free.
4. Dulbecco's Modified Eagle Medium—high glucose with L-glutamine and sodium pyruvate (DMEM-hg).
5. Dulbecco's Modified Eagle Medium–low glucose (DMEM-lg).
6. 0.5 % Trypsin EDTA/TrypLE™ Express.
7. Fetal Bovine Serum (FBS, heat-inactivated).
8. Embryonic stem cell-qualified FBS (ES-FBS).
9. Dimethyl Sulfoxide (DMSO).
10. 70 % Ethanol.
11. Penicillin/Streptomycin (PenStrep) 100×.
12. Non-Essential Amino Acids (NEAA) 100×.
13. L-Glutamine 100×.
14. β-mercaptoethanol, 55 mM.
15. 0.1 % gelatin.
16. Leukemia Inhibitory Factory (LIF).
17. Tryphan blue.
18. Primary and secondary antibodies (*see* **Note 2**).
19. 3 % paraformaldehyde (PFA).
20. Mitomycin C.
21. Bovine serum albumin solution (BSA) (*see* **Note 3**).
22. Antibody Dilution Buffer (1× PBS/1 % BSA /0.3 % Triton ×100).

2.4 Media Solutions (If Required Filter Through 0.2 μm Filters)

1. MSC medium: DMEM-lg, 15 % FBS, 1× PenStrep, 10 μL/mL from 55 mM (stock) β-mercaptoethanol (*see* **Note 4**).
2. MSC differentiation (Diff) medium: DMEM hg1× NEAA10 μl/mL from 55 mM (stock) β-mercaptoethanol1×penstrep10 % FBS.

3. Freezing medium (hiPSC): PSC cryopreservation medium (Life Technologies).
4. Freezing medium (MSC): 50 % MSC medium, 40 % ES-FBS, 10 % DMSO.

3 Methods

3.1 hiPSC Maintenance (Manual Passaging) (See Note 2)

1. hiPSCs usually grow in colonies. When plated onto a Geltrex-coated plate, they look like pancakes (Fig. 1a) (*see* **Note 3**).
2. Supplement daily with Essential 8 medium (*see* **Note 4**).
3. The colonies are allowed to grow for 4–5 days. When the confluency has reached 80 %, the cells need to be passaged.
4. Passaging is carried out by gridding the colonies using a sterile needle (Fig. 1b) (*see* **Note 5**). These pieces are then gently lifted using a pipette tip or cell lifters.
5. The resultant pieces are collected and plated onto a new Geltrex-coated plate.
6. For freezing, the pieces need to be pelleted at 200 × *g* for 10 min. Resuspend the pieces very gently in the PSC medium in a cryovial.
7. Regular characterization of the hiPSC needs to be done. This can be carried out by immunostaining.

3.2 hiPSC Differentiation to MSCs (Fig. 2)

1. When the hiPSCs have reached the desired confluency, wash the colonies using PBS.
2. Remove spontaneously differentiated areas.
3. Add differentiation medium to the plate.
4. In a very gentle manner, lift the whole colonies off the plate using a cell lifter (*see* **Note 6**).
5. Place these floating colonies in low attachment plates. One 6 cm dish to one well of the 6-well plate. Add sufficient medium to last for 3 days (*see* **Note 7**).
6. These suspended colonies will ball up in a day and form embryoid bodies (EBs) (Fig. 1c) (*see* **Note 8**).

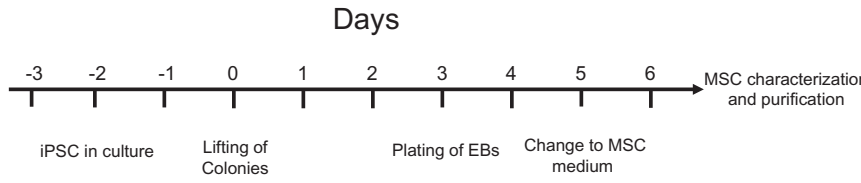

Fig. 2 Schematic timeline of the hMSC differentiation from hiPSC

7. On day 3, plate the EBs onto a gelatin-coated 6-cm plate.
8. Supplement with MSC medium.
9. Thereafter, observe the EBs daily to check if they have flattened and cells start growing out of them (Fig. 1d).

3.3 Enriching MSCs

1. Trypsin treatment needs to be carried out in multiple stages.
2. Stage 1–trypsin treatment for 1 min followed by a quick rinse with PBS to wash away non-MSC cell types.
3. Stage 2–reintroduce trypsin, for 2–3 min to help detach MSCs.
4. Plate the cells in a new dish for a couple of hours. MSCs tend to attach quickly. Afterward, very gently, wash the dish surface with MSC medium and discard the wash (*see* **Note 9**).
5. These steps will successively allow a greater number of MSCs to populate the plate.
6. Once immunostaining has confirmed the presence of MSCs, they can be purified through FACS system.

3.4 Immunostaining of hiPSC and MSCs

1. Antibodies for hiPSC—Oct4a, SOX2, SSEA4, Nanog, Tra-1-81, Tra-1-60.
2. Antibodies for MSC—CD 44 (+), CD 29 (+), CD 45 (−), and CD 34 (−).
3. Grow the cells on cover slips (*see* **Note 10**).
4. Fix the cells in 4 % PFA for 15 min.
5. Block the cells in desired blocking buffer for 60 min.
6. While blocking, prepare primary antibody by appropriate dilution in antibody dilution buffer.
7. Aspirate blocking solution, apply diluted primary antibody.
8. Incubate overnight at 4 °C.
9. Rinse three times in 1× PBS for 5 min each.
10. Incubate specimen in secondary antibody diluted in Antibody Dilution Buffer for 1–2 h at room temperature in the dark.
11. Rinse three times in 1× PBS for 5 min each.
12. Mount and observe under fluorescence microscope (Figs. 3 and 4).

4 Notes

1. It is better to avail most of these commercially to simplify quality assessment and avoiding batch variability.
2. hiPSCs can be cultured enzymatically.

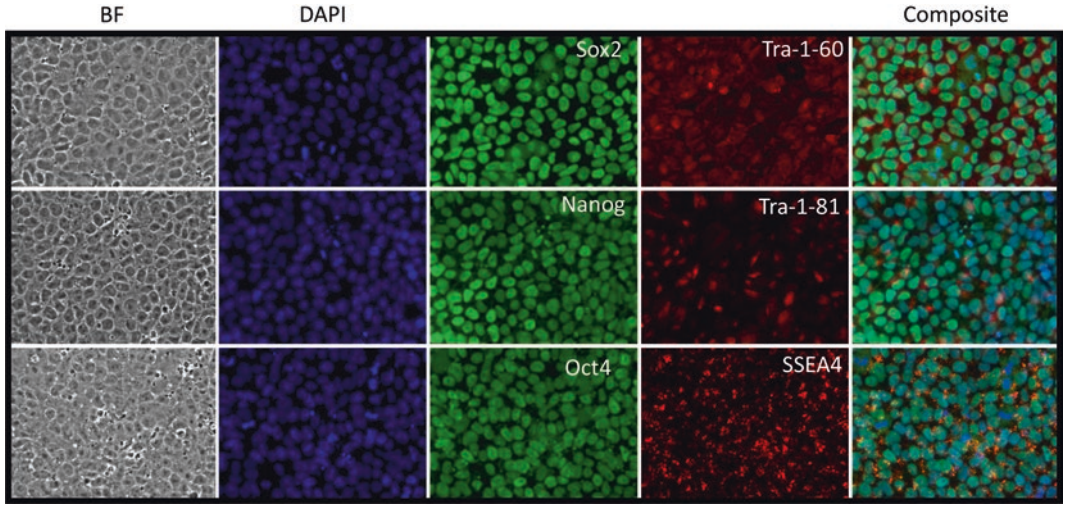

Fig. 3 Immunofluorescence staining of hiPSC markers at 40X

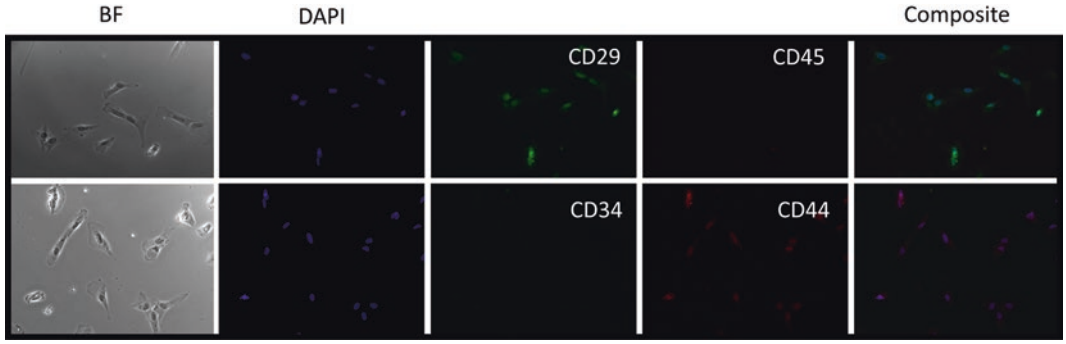

Fig. 4 Immunofluorescence staining for hMSC markers at 20X

3. hiPSC can be cultured on other matrices like matrigel, and vitronectin. Further, they can also be cultured as single cells.
4. Homemade medium consisting of DMEM/F12 may be used too.
5. When using enzyme, a serological pipette can be used to grid the whole plate and break them to pieces.
6. If the lifting shreds the colonies or if there is excessive death, the cells may be treated with 1 U/mL Dispase. This helps in easier lifting of the colonies.
7. Alternatively, the medium can be changed every day.
8. If EB formation is not satisfactory. ROCK inhibitors can be added. The optimum concentration needs to be adjudged through trial and error.

9. As MSCs tend to attach quickly, other cells like fibroblasts can be washed away.
10. Any other desired format may be used, like chamber slides, glass bottomed dishes, etc.

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Chapter 15

A Simplified and Systematic Method to Isolate, Culture, and Characterize Multiple Types of Human Dental Stem Cells from a Single Tooth

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Abstract

This chapter describes a simplified method that allows the systematic isolation of multiple types of dental stem cells such as dental pulp stem cells (DPSC), periodontal ligament stem cells (PDLSC), and stem cells of the apical papilla (SCAP) from a single tooth. Of specific interest is the modified laboratory approach to harvest/retrieve the dental pulp tissue by minimizing trauma to DPSC by continuous irrigation, reduction of frictional heat from the bur rotation, and reduction of the bur contact time with the dentin. Also, the use of a chisel and a mallet will maximize the number of live DPSC for culture. Steps demonstrating the potential for multiple cell differentiation lineages of each type of dental stem cell into either osteocytes, adipocytes, or chondrocytes are described. Flow cytometry, with a detailed strategy for cell gating and analysis, is described to verify characteristic markers of human mesenchymal multipotent stromal cells (MSC) from DPSC, PDLSC, or SCAP for subsequent experiments in cell therapy and in tissue engineering. Overall, this method can be adapted to any laboratory with a general setup for cell culture experiments.

Key words Dental Pulp Stem Cells (DPSC), Stem Cells from Apical Papilla (SCAP), Periodontal Ligament Stem Cells (PDLSC), Mesenchymal Stromal Cells (MSC), Lineage differentiation, Flow cytometry, Cell differentiation, Dental pulp, Periodontal ligament, Periapical tissue

1 Introduction

Multipotent mesenchymal stromal cells (MSCs) are the most often used cell population in tissue engineering because of its multilineage potential from multiple sources and its ability for self-renewal [1–3]. Commonly used MSCs are bone marrow-derived mesenchymal stem cells (BM-MSC), and adipose tissue-derived stem cells (ADSC). Among various sources, dental stem cells are a promising source of MSCs. In addition, dental stem cells fulfill the minimum requirements of the International Society for Cellular Therapy (ISCT) for Human MSC [4]. These criteria include: (a) plastic

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adherence, (b) expression of positive antibody markers CD105, CD73, and CD90, and lack of expression of the negative antibody markers CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR, and (c) MSCs demonstrate multilineage differentiation.

Dental MSCs are less invasive to harvest (ease of access), can be cryopreserved, possess a higher cell proliferation rate and survival time than BM-MSC [5]. Also, human dental stem cells have been used for bone regeneration in critical size defects [5], promoting osseointegration of dental implants [6], treating spinal cord injury [7], accelerating skin wound healing [8], increasing angiogenesis in ischemic hind limb [9], improving cardiac function, and reducing infarct size in myocardial infarction [10].

Human dental pulp stem cells (DPSC) were first isolated by Gronthos and colleagues in 2000 [11]. The isolation of periodontal ligament stem cells (PDLSC) [12] and stem cells from the apical papilla (SCAP) soon followed [13]. In the last decade, various methods were described for the isolation of human dental stem cells. However, each method focused on one type of dental stem cells. In this chapter, we have combined these previously reported methods into a simplified, unified, and systematic approach to isolate multiple types of dental stem cells from a single tooth. Notably, we recommend a gentle technique to retrieve the dental pulp with reduced trauma to DPSC by continuous irrigation, reduction of frictional heat from the bur rotation, and reduction of the bur contact time with the dentin. In addition, the use of a chisel and a mallet will maximize the number of live DPSC. Additionally, this chapter describes the cell isolation, characterization by flow cytometry, and multilineage differentiation of DPSC, PDLSC, and SCAP (Fig. 1). Overall, the benefits of the described approach include maximizing the cell number from multiple dental cell sources from one tooth, cost reduction, and time saving.

2 Materials

2.1 Isolation and Primary Culture of Stem Cells of Apical Papilla (SCAP), Periodontal Ligament Stem Cells (PDLSC), and Dental Pulp Stem Cells (DPSC)

1. 15 or 50 ml conical tube.
2. Phosphate Buffered Saline (PBS without Ca^{++} and Mg^{++}), pH 7.4.
3. Sterilized gauze.
4. 60-mm or 100-mm tissue culture dish.
5. Periodontal scaler.
6. Scalpel with a #15 blade.
7. High-speed handpiece.
8. Fissure bur 701 or 558.
9. Chisel.

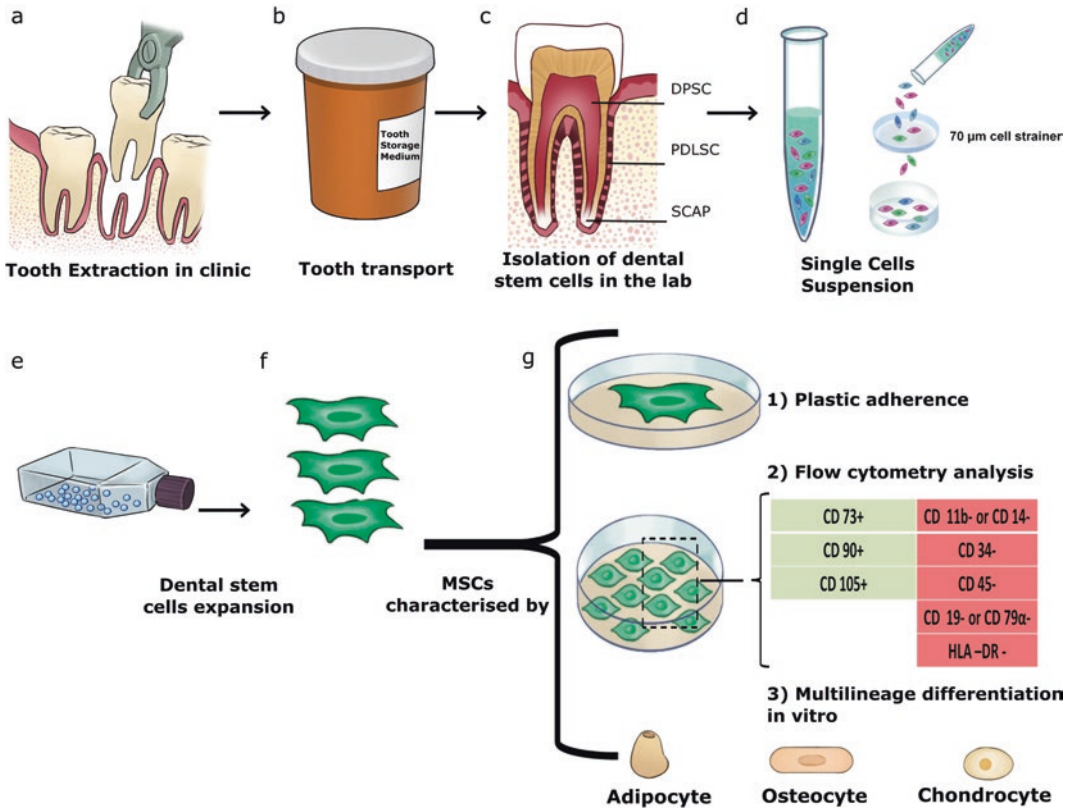

Fig. 1 Diagrammatic representation of the dental stem cell isolation and characterization steps. (**a**, **b**) Extracted teeth from medically healthy patients are transferred to a container with a storage solution. (**c**) In the laboratory, the isolation of stem cells of apical papilla (SCAP), periodontal ligaments stem cells (PDLSC), and dental pulp stem cells (DPSC) is performed. (**d**) A single cell suspension is obtained after enzymatic digestion of the tissue, and then filtered through a 70-µm cell strainer to remove large cell clumps and tissue debris. (**e**, **f**) Dental stem cells are expanded in a cell tissue culture or flask to generate sufficient cells. (**g**) The main features of mesenchymal stromal cells (MSCs) are their: (1) Plastic adherence; (2) Expression of CD 73, CD 90, and CD 105, and lack of expression of CD 45, CD 34, CD 14 or CD 11b, CD 19 or CD 79α and HLA-DR; (3) In-vitro differentiation under specific cultured conditions into adipocytes, chondrocytes, and osteocytes

10. Mallet.
11. Micro tweezers.
12. Curved Micro Scissors.
13. Tooth extraction Forceps.
14. 70-µm cell strainer.
15. Ice bucket.
16. T-25 or T-75 Culture flask.
17. Tooth Storage Medium: DMEM-Dulbecco's Modified Eagle Medium or HBSS-Hank's Balanced Salt Solution, no calcium, no magnesium with 100 U/ml of penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (*see* **Note 1**).

18. Washing Solution: Phosphate buffered saline (PBS) with 2% antibiotic/Antimycotic (200 U/ml of penicillin, 200 μg/ml streptomycin, and 0.50 μg/ml amphotericin B) (*see* **Note 1**).
19. Complete growth medium: α-MEM-alpha Minimum Essential Medium, supplemented with 15% (v/v) FBS-Fetal Bovine Serum, 0.1 mM l-ascorbic acid phosphate, and 1% antibiotic/antimycotic (100 U/ml of penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) (*see* **Note 1**).
20. Digestion enzyme solution: 4 mg/ml Dispase II, 3 mg/ml Collagenase type 1, and 1% antibiotic/antimycotic in PBS (*see* **Notes 2** and **3**).
21. 2, 5, and 10 ml sterile pipettes.

2.2 Cell Counting

1. Trypan blue stain 0.4%.
2. Neubauer counting chamber.
3. Glass cover slip 22 × 22 mm.
4. Hand tally counter.

2.3 Cell Passage

1. Trypsin–EDTA 0.25%.
2. Phosphate Buffered Solution (PBS) without Ca^{++} and Mg^{++}.
3. Complete Growth Medium.

2.4 Cell Cryopreservation and Recovery

1. Cryovials (1.8 ml).
2. Freezing medium solution: 90% FBS with 10% Dimethyl sulfoxide.
3. 0.25% Trypsin–EDTA.

2.5 Multilineage Differentiation

2.5.1 Osteogenic Differentiation

1. Osteoblast differentiation medium: α-MEM, 1% antibiotic/antimycotic (100 U/ml penicillin-G, 100 μg/ml streptomycin, and 0.25 μg/ml Amphotericin B), supplemented with 2% FBS, 50 μg/ml ascorbic acid, and 10 $^{-8}$ M dexamethasone 10 mM β-glycerophosphate (*see* **Note 4**).
2. Alizarin Red S solution: 1% Alizarin red S in distilled water.
3. 70% ethanol.
4. PBS (without Ca^{++} and Mg^{++}).
5. Distilled water.

2.5.2 Adipogenic Differentiation

1. Adipogenic Induction Medium (Lonza): insulin, l-glutamine, mesenchymal stem cell growth supplement (MCGS), dexamethasone, indomethacin, 3-isobuty-l-methyl-xanthine (IBMX), gentamicin amphotericin-B (GA) 1000.
2. Adipogenic maintenance medium (Lonza): insulin, l-glutamine, MCGS, GA-1000.

3. Oil red O stain: 0.3 g oil red O stain dissolved in 100 ml isopropanol.
4. PBS (without Ca^{++} and Mg^{++}).
5. 10% Neutral Buffered Formalin.
6. 60% Isopropanol.
7. Distilled water.

2.5.3 Chondrogenic Differentiation

1. StemXVivo Chondrogenic Base Media (R&D Systems): add 1% antibiotic/antimycotic (100 U/ml penicillin-G, 100 µg/ml streptomycin, and 0.25 µg/ml Amphotericin B).
2. StemXVivo Chondrogenic Supplement (R&D Systems).
3. Alcian Blue solution: 1% alcian blue in 3% acetic acid, pH 2.5.
4. 4% paraformaldehyde.
5. Distilled water.
6. 3% acetic acid.

2.6 Flow Cytometry: Sample Preparation

1. Viability Marker FVS450 (*see* **Note 5**).
2. Stain buffer (FBS).
3. Fc Receptor Block.
4. 12 × 75 mm polypropylene tubes (5 ml round-Bottom Tube).
5. Accutase cell detachment solution.
6. 70-µm cell strainer.
7. PBS (without Ca^{++} and Mg^{++}).

2.7 Flow Cytometry Antibodies

hMSC analysis kit (BD Stemflow ™, BD Biosciences), includes:

1. FITC Mouse Anti-Human CD90 (Clone 5E10, BD Biosciences).
2. PE Mouse Anti-Human CD44 (Clone: G44-26; BD Biosciences).
3. PerCP-Cy™5.5 Mouse Anti-Human CD105 (Clone: 266; BD Biosciences).
4. APC Mouse Anti-Human CD73 (Clone: AD2; BD Biosciences).
5. hMSC Positive Isotype Control Cocktail: (*mIgG1, κ FITC (Clone: X40); mIgG1, κ PerCP-Cy5.5 (Clone: X40); mIgG1, κ APC (Clone: X40); BD Bioscience)*.
6. PE hMSC Negative Isotype Control Cocktail*: (mIgG1, κ PE (Clone: X40); mIgG2a, κ PE (Clone: G155-178); BD Biosciences)*.
7. hMSC Positive Cocktail *(CD90 FITC (Clone: 5E10); CD105 PerCP-Cy5.5 (Clone: 266);CD73 APC (Clone: AD2); BD Biosciences)*.
8. PE hMSC Negative Cocktail (20 µl) *(CD34 PE (Clone: 581); CD11b PE (Clone: ICRF44);CD19 PE (Clone: HIB19); CD45 PE (Clone: HI30); HLA-DR PE (Clone: G46-6); BD Biosciences)*.

3 Methods

3.1 Teeth Selection Criteria

Teeth selected from patients with a healthy medical history and having the following criteria:

1. Erupted third molars or orthodontically extracted permanent premolars with a normal dental pulp and normal apical tissues are recommended for the isolation of DPSC, PDLSC.
2. Teeth with an immature root, normal dental pulp, and normal apical tissues are recommended for the isolation of SCAP.

3.2 Isolation of SCAP, PDLSC, and DPSC

This section describes steps to isolate the dental pulp stem cells (DPSC), periodontal ligament stem cells (PDLSC), and stem cells from apical papilla (SCAP) from a single tooth (Fig. 2).

1. Following informed consent, collect the extracted teeth from patients and store in 50 ml of tooth storage solution at 4 °C for up to 24 h (*see* **Note 6**).
2. Aspirate the storage solution and rinse twice with washing solution until the removal of blood and debris.
3. Place tube with the teeth in the ice bucket, and then transfer one tooth at a time to a 35 mm dish.
4. Excise and discard any gingival tissues using scalpel and scissors (Fig. 2b).
5. SCAP isolation: wash teeth with 10 ml of autoclaved PBS three times, and then hold the crown portion of the tooth with a sterile gauze. Collect the apical papilla tissue on the outer surface of the immature root using a surgical blade. Direct the surgical blade at the level of the immature root apex, and then collect tissue from the outer surface of the root. Transfer the apical papilla to another cell culture dish with washing solution.
6. PDLSC isolation: Use a tweezer to stabilize the tooth in the cell culture dish with small amount of washing solution. Use a surgical blade #11 or a periodontal scaler to gently scrape and discard the PDL tissues surrounding the tooth at the CEJ (cemento-enamel junction) and coronal third level of the root. Collect and keep PDL tissues from the middle third of the root toward the apex of the tooth (*see* **Note 7**, Fig. 2c).
7. DPSC isolation: a modified technique [11] is used to isolate DPSC. Hold the crown portion of the tooth with a tooth extraction plier or with a sterile gauze (Fig. 2d). Use a high-speed handpiece with a straight fissure bur to create a horizontal groove at the CEJ level while irrigating with washing solution until pulp chamber is exposed (fig. 2e) (*see* **Notes 8** and **9**). Transfer the tooth and wrap it with sterile gauze. Then

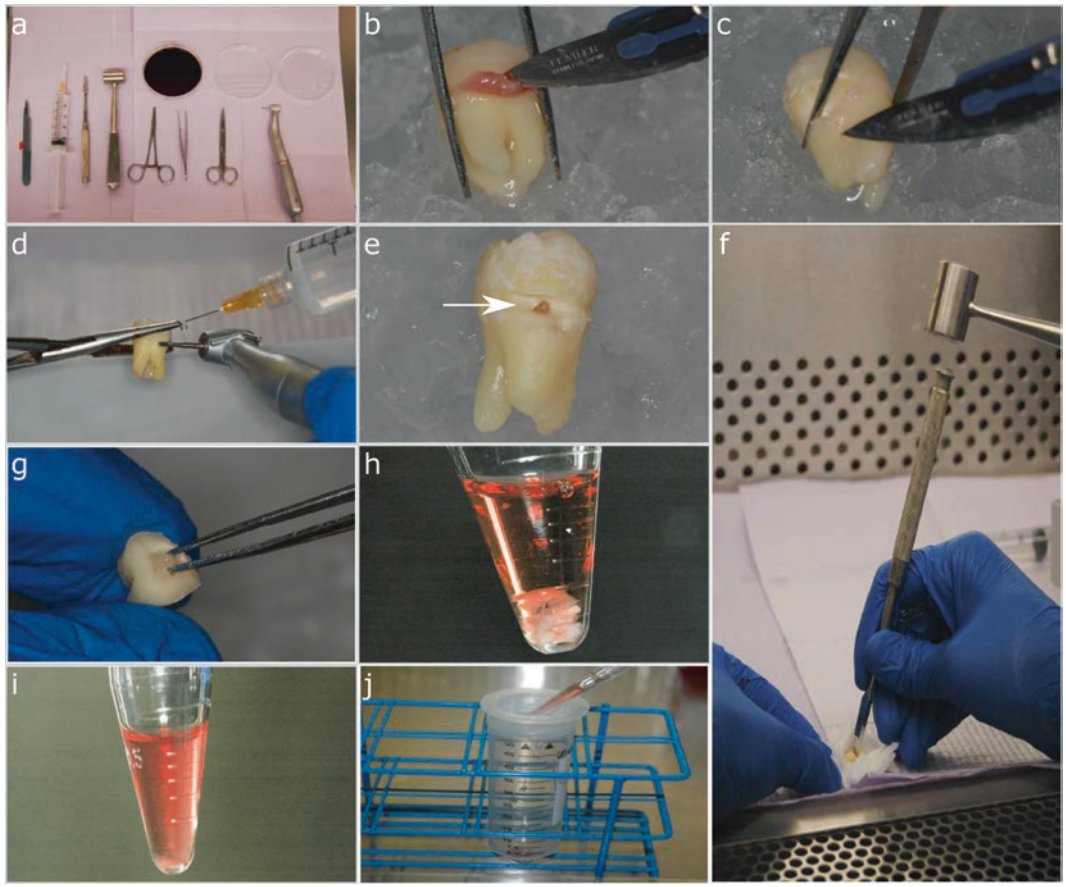

Fig. 2 Steps to isolate dental pulp stem cells (DPSC) and periodontal ligament stem cells (PDLSC) from a single tooth. (**a**) Basic armamentarium for dental stem cells isolation. (**b**) Surgical blade #11 is used to excise gingival tissue from a tooth inside a cell tissue culture dish placed on the top of a container filled with ice. (**c**) Surgical blade #11 is used to gently scrape the periodontal tissue from the middle third of the root toward root apex. (**d**) Tooth held with a hemostat and a horizontal groove is created at the cemento-enamel junction (CEJ) with a straight fissure carbide bur while irrigating with sterile PBS. (**e**) A horizontal groove is created at the depth of the pulp chamber (the white arrow shows the pulp chamber and pulpal tissue exposed). (**f**) Tooth split by using a chisel and mallet. (**g**) Dental pulp tissue extracted by micro-tweezers. (**h**) Dental pulp tissue digested with dispase II, collagenase type 1, and 1% antibiotic/antimycotic in PBS. (**i**) Digested dental pulp tissue after 1-h incubation at 37 °C. (**j**) Solution containing DPSC poured through a 70-μm cell strainer to remove any large tissue remnants or cell clumps

split the tooth by placing a chisel in the created groove and a mallet to split the tooth (Fig. 2f) (*see* **Notes 10** and **11**). Pull out the pulp tissue using sterile micro tweezers, and transfer the extracted pulp tissue into another sterile cell culture dish with washing solution (Fig. 2g).

8. Mince the DPSC or SCAP tissues into 2-4 mm pieces with a surgical blade and/or surgical scissors.

9. Transfer each dental stem cell groups into a pre-labeled 50 ml tube (such as SCAP, PDLSC, and DPSC). Centrifuge tissues at 400 × *g* for 5 min, 4 °C and discard the supernatant.
10. Add the enzyme solution (4 ml/tube) to the cell pellet; incubate for not more than 60 min in a 37 °C shaker. Stop procedure when tissues are digested (Fig. 2h, i) (*see* **Notes 12** and **13**).
11. Add 4 ml of complete growth medium to inhibit the enzyme digestion (*see* **Note 14**).
12. Centrifuge at 400 × *g* for 5 min, 4 °C, discard supernatant, and resuspend with culture medium 4 ml.
13. Filter the cell suspension with a 70-μm cell strainer (Fig. 2j), centrifuge and discard the supernatant.
14. Resuspend the cell pellet with 4 ml of complete growth medium.
15. Count cells (*see* Subheading 3.3).
16. Seed cells at a density of 3×10^4 cells/cm^2 in complete growth medium to generate primary cultures.
17. Change medium every 3–4 days until cells are 60–70% confluent.

3.3 Cell Counting

1. Take 10 μl of cell suspension and add 10 μl of 0.4% trypan blue solution, to highlight nonviable cells.
2. Place cover slip 22 × 22 mm on the Neubauer chamber central area. Adjust the micropipette to aspirate 10 μl.
3. Place pipette tip close to the cover slip edge, right at the center of the Neubauer chamber. Release the plunger slowly watching how the liquid enters the chamber uniformly absorbed by capillarity.
4. Place the Neubauer chamber on the microscope stage. Count cells in the four-grid square, write the cells counted for all single square, and repeat count in triplicate for all samples. Obtain cell concentration using the general formula:

$$\frac{\text{Total cells} / ml : \text{number of cells} \times 2\left(\text{dilution factor}\right) \times 10000 \text{ cells} / ml}{\text{Number of squares}}$$

$$\frac{\text{Total cell number} : \text{number of cells} \times 2\left(\text{dilution factor}\right) \times 10000 \text{ cells} / ml \times \text{volume}\left(ml\right)}{\text{Number of squares}}$$

3.4 Cell Passage

1. Aspirate culture medium when cells are 60-70% confluent from the culture plates (passage 0, "P0").
2. Wash flasks or plates with PBS. Aspirate the PBS.

3. Add cell-detaching solution 0.25% Trypsin–EDTA. Place culture dish in an incubator at 37 °C for 2–5 min until all cells have detached. Detached cells should look round, plump, and refract light around their membrane; some cells may clump.
4. Add culture medium to inhibit trypsinzation, and then transfer cell solution to 15 or 50 ml conical tube. Centrifuge at 400 × *g* for 5 min. Aspirate medium at the bottom of the conical tube being careful not to disrupt the cell pellet.
5. Resuspend the cell pellet in a new, pre-warmed, complete growth culture medium by gently pipetting up and down to obtain a single-cell suspension (*see* **Note 15**).
6. Perform cell count and reseed into culture flasks at a plating density of 1×10^4 cell/cm^2.

3.5 Cell Cryopreservation and Recovery

1. Harvest cells when at 60–70% confluence (*see* **Note 16**, and Subheading 3.4).
2. Resuspend cells with freezing medium solution of 1-2 × 10^6 cells per ml.
3. Add 1 ml of cells into labeled 1.8 ml cryovials, and then freeze at a rate of 1 °C/min using a cryo 1 °C freezing container "Mr. Frosty" filled with isopropanol precooled to 4 °C.
4. Place the container holding the cryovials at −80 °C overnight before transferring the cryovials into liquid nitrogen for long-term storage.
5. Thaw cells in 37 °C in a water bath to recover cryopreserved stock (*see* **Note 17**).
6. Resuspend cells with complete growth medium and spin at 400 × *g* for 10 min.
7. Assess viability of cells using 0.4% trypan blue/PBS (*see* Subheading 3.3). Typically, this procedure results in cell viabilities between 80 and 90%.

3.6 Multilineage Differentiation

This section describes the osteogenic, adipogenic, and chondrogenic differentiation for dental stem cells (Fig. 3).

3.6.1 Osteogenic Differentiation

1. Seed 4.2 × 10^3 cells/cm^2 in vitro-expanded cells per well using a 24-well plate or 48-well plate in culture growth medium, and incubate at 37 °C in 5% CO_2 and >90% humidity.
2. Cells should be 50–70% in 1–2 days.
3. Aspirate culture growth medium at 50–70% cell confluence, and add an equivalent volume of osteogenic inductive medium.
4. Replace the osteogenic inductive medium every 3–4 days.
5. After 4 weeks, aspirate the medium and gently rinse the osteogenic-induced culture once with PBS and fix cells in 70% ethanol at −20 °C for 1 h, rinse cells with distilled water.

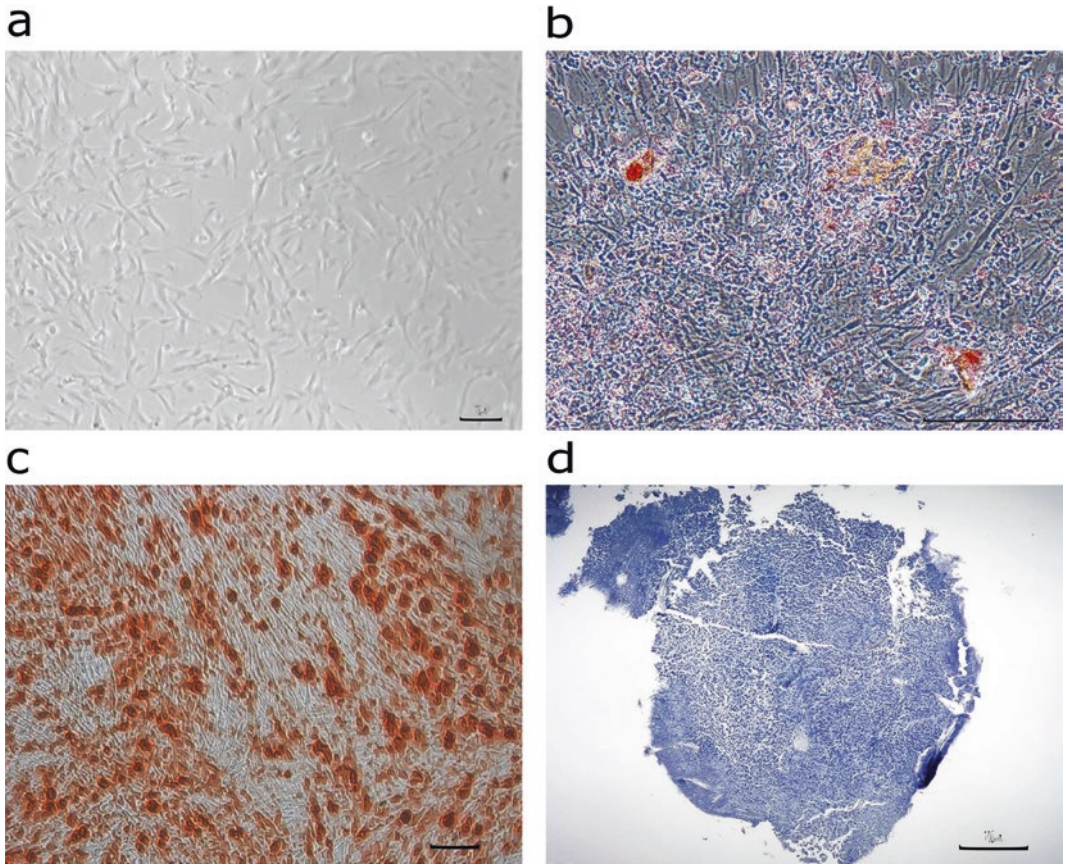

Fig. 3 Dental MSCs morphology and multilineage differentiation. (**a**) Morphology of cultured DPSC at passage 3. All MSCs exhibited spindle-shaped morphology. Scale bar = 130 μm. (**b**) PDLSC differentiation in adipogenic induction medium for 4 weeks. Adipocytes stained with Oil Red O. Scale bar = 100 μm. (**c**) DPSC osteogenic differentiation in osteogenic induction medium for 4 weeks, stained with Alizarin Red stain. Scale bar = 180 μm. (**d**) DPSC chondrogenic differentiation in chondrogenic induction medium for 4 weeks, stained with Alcian blue stain. Scale bar = 150 μm

6. Stain the osteogenic-induced culture with Alizarin Red S stain for 10 min at room temperature.
7. Wash five times with distilled water to remove excess stain.
8. Wash with PBS to reduce nonspecific staining for 15 min (Fig. 3c).

3.6.2 Adipogenic Differentiation

1. Seed 2.1×10^4 cells/cm^2 in-vitro expanded cells per well using a 24-well plate in culture growth medium and incubate at 37 °C in 5% CO_2 and >90% humidity, cells should be 100% in 3–4 days.
2. At 100% confluence, three cycles of induction/maintenance will stimulate optimal adipogenic differentiation. Aspirate the culture growth medium and add an equivalent volume of

adipogenic inductive medium. Each cycle consists of feeding the MSC with supplemented adipogenesis induction medium and culture for 3 days at 37 °C in 5% CO2, followed by 1–3 days of culture in supplemented adipogenic maintenance medium (*see* **Notes 18** and **19**).

3. Replace the adipogenic inductive medium every 2–3 days (*see* **Note 20**).
4. After 3 complete cycles of induction/maintenance, culture the MSC for 7 more days in the supplemented adipogenic maintenance medium, replacing the medium every 2–3 days.
5. After 4 weeks, aspirate the medium and gently rinse the adipogenic-maintenance medium once with PBS. Aspirate the PBS. Add 10% neutral buffered formalin to cover the cell monolayer. Incubate at room temperature for at least 30 min.
6. Aspirate the fixation buffer and wash the cell monolayer with distilled water. Carefully aspirate the water and add enough 60% isopropanol to cover the cells. Incubate at room temperature for 5 min.
7. Carefully aspirate 60% isopropanol and add enough 0.3% Oil Red O staining solution to cover the cells monolayer. Incubate at room temperature for 15 min (*see* **Note 21**).
8. Aspirate the Oil Red O stain and wash several times with distilled water to remove excess stain until the water becomes clear.
9. Aspirate the water, blot the plates on a paper towel to remove as much water as possible, and then add PBS on the plates (Fig. 3b).

3.6.3 Chondrogenic Differentiation

1. Seed cells between 2.5×10^5 and 1×10^6 cells in 15 ml polypropylene tube.
2. Centrifuge the cells at 400 × *g* for 5 min, 4 °C. Remove the supernatant and resuspend the cells with 0.5 ml chondrogenic medium (*see* **Note 22**).
3. Centrifuge the cells at 400 × *g* for 5 min, 4 °C. Do not remove the medium. Loosen the cap of the tube to allow gas exchange, and incubate at 37 °C and 5% CO_2.
4. After 1–2 days, the cell pellet will form a round ball approximately 1–2 mm in diameter. Cell pellet remains the same size for the entire culturing time.
5. Every 2–3 days, carefully remove and discard the supernatant and replace with 0.5 ml of chondrogenic medium (*see* **Note 23**).
6. Harvest chondrogenic pellet after 14–28 days in culture.
7. Aspirate chondrogenic medium and wash twice with PBS.

8. Fix cell pellet in 4% paraformaldehyde overnight, then embed in paraffin, and cut to 5 μm sections from block and mount on glass slide.
9. Deparaffinize slides and rehydrate to distilled water.
10. Immerse slide in 3% acetic acid for 3 min.
11. Stain in alcian blue solution for 30 min.
12. Wash in running tap water for 2 min. Rinse in distilled water.
13. Mount the tissue sections and check under microscope (Fig. 3d).

3.7 Flow Cytometry: Sample Preparation

The following flow cytometry protocol is used to characterize the cell surface phenotype of the DPSC, PDLSC, and SCAP cell populations:

1. MSC cells are passaged until a cell count of 5–10 × 10^6 cells/ml is obtained (*see* **Note 24**).
2. Remove tissue culture medium from the plate or flask and wash the cells with PBS (without Ca^{++} or Mg^{++})
3. Detach cells by using Accutase Cell Detachment Solution for 5 min in a CO_2 incubator and then add culture medium to stop trypsinization (*see* **Note 25**).
4. Transfer cells into a conical tube (15 or 50 ml), centrifuge for 5 min, 400 × *g* at 4 °C, discard supernatant, resuspend with PBS, filter through 70 μM cell strainer, and perform a cell count and viability analysis using trypan blue.
5. Label 8 polypropylene tubes 1–8; add the antibodies mixes as shown below:

   Tube 1: Cells only, no antibody.

   Tube 2: Cells with the viability marker.

   Tube 3: FITC Mouse Anti-Human CD90 (5 μl).

   Tube 4: PE Mouse Anti-Human CD44 (2.5 μl).

   Tube 5: PerCP-Cy™5.5 Mouse Anti-Human CD105 (5 μl).

   Tube 6: APC Mouse Anti-Human CD73 (5 μl).

   Tube 7: hMSC Positive Isotype Control Cocktail (20 μl) and PE hMSC Negative Isotype Control Cocktail (20 μl).

   Tube 8: hMSC Positive Cocktail (20 μl) PE hMSC Negative Cocktail (20 μl).

   Keep tubes in the dark (*see* **Note 26**).
6. Place approximately 1 × 10^6 cells in tube number 1.
7. The remaining cell suspension (approximately 7 × 10^6 cells) is transferred to a 15 ml conical tube.

8. Add Fixable Viability Stain FVS450 (1:1000 dilution in PBS), vortex immediately.
9. Incubate the mixture for 15 min at 4 °C (in the dark).
10. Wash cells twice with 2 ml of Stain Buffer and resuspend cells with Stain Buffer.
11. Add Fc Receptor block to the cell suspension (1:5 dilution). Total amount of Fc receptor block: *7 × 20 μl = 140 μl* (*see* **Note 27**), and add 20 μl to tube 1. Keep on ice, in the dark, for 20 min.
12. Add 100 μl cell suspensions (approximately 1×10^6 per tube) to tubes 2-8.
13. Incubate tubes on ice, in the dark, for 30 min.
14. Wash the cells twice with Stain Buffer (add 2 ml of buffer per tube, centrifuge and repeat), resuspend in 300 μl in Stain Buffer.
15. Analyze cells using a flow cytometer analyzer equipped with a 405, 488, and 640 nm lasers and at least these seven detectors (405 nm: 450 BP; 488 nm: 530 BP, 585 BP, 695 BP, FSC, SSC; 640 nm: 660 BP).

3.8 Flow Cytometry: Gating Strategy and Analysis

This section describes the gating strategy for the flow cytometry analysis. The gating strategy is used to select the cell population of interest and minimize binding of nonspecific antibodies from debris, dead cells, and cell clumps. In the experiment, tubes 1–6 are used as compensation controls and to adjust the cytometer's PMT detectors, and tubes 7–8 are used as test samples.

Data was recorded on three lasers, 11 detectors LSR Fortessa equipped with BD FACS Diva Software (v6, BD Biosciences). Postacquisition analysis was performed using FlowJo.

1. Place the unstained control (Tube 1) on the flow cytometer and adjust the optimal detector voltages to achieve maximal signal-to-background ratio. In order to isolate single cells from doublets, record the Height and Width values for the FSC and SSC parameters.
2. Place compensation controls tube 2–6 on the flow cytometer. Record a minimum of 30,000 events per compensation sample, for each sample, select the positive and negative peaks (*see* **Note 28**).
3. Once compensation controls are collected, the software automatically calculates the compensation.
4. Place sample tubes 7–8 on the flow cytometer. Record a minimum of 100,000-events per sample tube.
5. View the MSC population in the side scatter area (SSC-A) versus forward scatter area (FSC-A) (Fig. 4a).

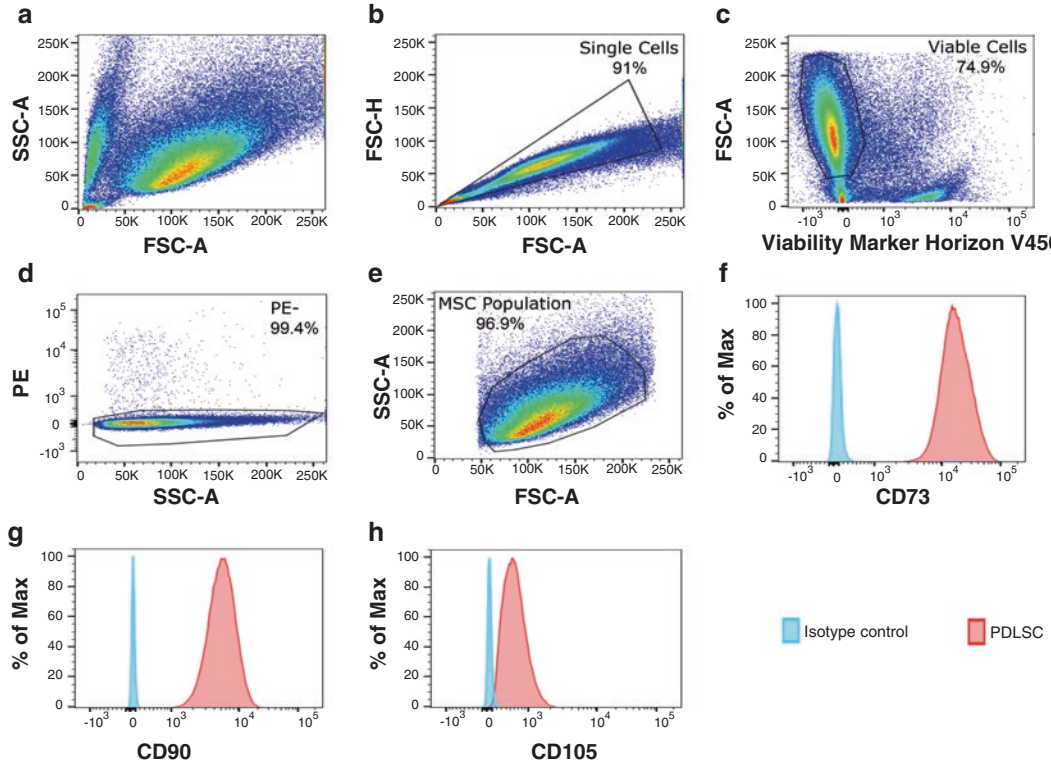

Fig. 4 The gating strategy and immunophenotype analysis of mesenchymal stromal cells (MSCs) from periodontal ligament stem cells (PDLSC). PDLSCs at passage 5 were detached with Accutase, labeled with antibodies against the indicated antigens, and analyzed by flow cytometry. Gating strategy for the flow cytometry analysis (**a**–**e**). (**a**) Shows the total population of interest (FSC-A versus SSC-A). (**b**) Cells were first gated for single cells (FSC-A versus FSC-H). The gate is further analyzed for (**c**) their uptake of the viability stain to determine live versus dead cells (Viability marker vs. FSC-A). (**d**) The Negative cocktail-PE dump channel is gated out (SSC-A versus PE). (**e**) MSC population is gated (FSC-A versus SSC-A), positive antibody panel surface expression is then determined from this gated population. A histogram comparison between isotype control and samples is shown for the positive markers (**f**) CD 73, (**g**) CD 90, and (**h**) CD 105

6. Exclude clumped cells using a SSC-A versus SSC-H dot plot (Fig. 4b).
7. Select viable cells using a FSC-A versus Fixable Viability dot plots. Gate on events showing low fluorescence. Debris can be excluded by gating out low FSC-A events (Fig. 4c).
8. Gate out the negative cocktail-PE dump channel using a SSC-A versus PE dot plots (Fig. 4d).
9. The gate MSC population (Fig. 4e) is used to create single parameter histograms, each gated on the single antibodies CD73, 90, and 105 to measure the percentage of positive antibodies. The histograms of Isotype control (tube 7) are compared to samples (tube 8) (Fig. 4 f–h).

4 Notes

1. Prepare solutions in the laminar flow cabinet.
2. Prepare stock solution: 12 mg/ml Collagenase type I in PBS, 16 mg/ml Dispase II in PBS, DMEM with 1% antibiotic/antimycotic (100 U/ml of penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B). Prepare digestion enzyme solution by taking 1 ml Collagenase I, 1 ml Dispase II, and 2 ml DMEM with 1% antibiotic/antimycotic.
3. Total volume: 4 ml of digestion enzyme should be adequate for processing up to eight teeth.
4. Preparation of frozen stock aliquots of: ascorbic acid (50 mg/ml) in sterile PBS, dexamethasone 10^{-2} M in sterile deionized water, and β-glycerophosphate (1 M) in sterile deionized water and store at −20 °C. Prepare the osteoblast differentiation medium by diluting fresh aliquots of ascorbic acid and dexamethasone in α-MEM, 100 U/ml penicillin-G, 100 μg/ml streptomycin, supplemented with 2% FBS. Each culture is fed with fresh osteoblast differentiation medium. Prepare freshly mineralized medium on each culture feeding by adding fresh aliquots of β-glycerophosphate to the osteoblast differentiation medium.
5. FVS450 Stock Solution: Add FVS450 powder to 400 μl DMSO, and vortex the solution. Repeat vortexing to ensure powder dissolution. Aliquot into 100 μl and store at −20 °C. Discard solution after 40 days.
6. Perform all procedures in the biohazard laminar flow hood and wear sterile gloves to avoid contamination.
7. Avoid collecting any PDL tissues coronal to the middle third of the root to minimize bacterial contamination.
8. Repeat **steps 5**–7 for the other teeth from the same patient.
9. Create horizontal groove at the CEJ level either on the buccal, lingual, mesial, or distal surface of the tooth. At CEJ level, the pulp chamber is central and at a constant distance to the external surface of the tooth [14].
10. Use sterile gauze during splitting to avoid splattering of the tooth pieces.
11. The modified technique is used to minimize trauma to DPSC by continuous irrigation, reduction of frictional heat from bur rotation, and reduce bur contact time with dentin. We believe that the use of a chisel and a mallet will maximize DPSC numbers.
12. Vortex sample tube, every 10–15 min.
13. Avoid a prolonged digestion step to preserve cell viability.

14. Add culture medium in equal amount to the enzyme digestion solution.
15. Avoid bubble formation when resuspending the cell pellet.
16. Confirm absence of bacteria or fungi under the microscope.
17. Do not heat thawing cells to 37 °C. Remove the cryotube from the water bath as soon as the samples are thawed.
18. Adipogenic cells are delicate. Gently handle to avoid lipid vacuoles disruption.
19. Avoid cells to dry out when changing medium.
20. By day 4 after induction, small lip vacuoles should be visible. These vacuoles will reach their maximum size between 6 and 7 days postinduction. Mature adipocytes rapidly acidify the medium and require frequent change of the adipogenic medium (every 2 and 3 days).
21. Prepare Oil Red O staining solution: Dilute three parts of the Oil Red O stock solution with two parts of distilled water and filter the mixture with a syringe filter. Use within 30 min of preparation.
22. Chondrogenic medium: Add StemXVivo Chondrogenic Supplement (5 μl) to the completed StemXVivo Chondrogenic Base Media (495 μl) at a 1:100 dilution. This procedure will use 500 μl of chondrogenic medium for each 15 ml conical tube.
23. Use caution when removing the medium to avoid aspirating the pellet. Use a pipette to aspirate the medium. Avoid the use of a suction tip.
24. Ideal cell number: 10×10^6 cells/ml. Alternatively, cells can be resuspended at a concentration of 5×10^6 cells/ml, if cell number is a limiting factor.
25. Avoid the use of trypsin. Some antibody epitopes are affected by the enzyme treatment, such as the trypsin-sensitive epitope within human CD325 (N-cadherin) recognized by clone 8C11.
26. We recommend dye titration for optimal performance. It is important to maximize the signal while reducing the background signal as different cell types and different applications can result widely in staining.
27. Add 20 μl Fc Receptor block for the sample without the viability marker. $5–10 \times 10^5$ cells/tube into 12 × 75 mm plastic tubes.
28. In multicolor flow cytometry, a mathematical process known as compensation is used to remove spectral overlap between fluorochromes. Compensation is performed using a single compensation control sample labeled with each of the fluorochromes used in the experiment.

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Chapter 16

Measurement of Autophagy by Flow Cytometry

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Abstract

Autophagy activation is characterized by the accumulation of double-membrane autophagic vesicles (autophagosomes) in the cytoplasm. The mere presence of autophagosomes in the cytoplasm does not necessarily indicate an increased level of autophagy, since the blockade of any step downstream of autophagosome formation increases the number of autophagosomes. Therefore, quantitative methods for the detection of cytoplasmic protein turnover should be employed in addition to autophagosome monitoring, to verify increased levels of autophagy. At the present, multiple methods are available for the quantification of autophagy and the identification of autophagosomes. Here, we detail the in vitro methods currently available to detect autophagic cell death by flow cytometry analysis.

Key words Autophagosomes, Cell death, Flow cytometry, LC3, Lysosomes

1 Introduction

Autophagy is an evolutionarily conserved, homeostatic process responsible for degradation of both soluble proteins and organelles under stress conditions [1]. It involves the formation of double-membrane vacuoles (namely autophagosomes) that seize portions of cytoplasm and fuse with the lysosomes, thus forming the so-called autophagolysosomes [2]. Fusion brings the autophagocytosed material in contact with lysosomal enzymes, causing intralysosomal degradation. It is important to note that an increase of autophagosomes number is not a proof of increased autophagic activity. Autophagosome accumulation can be due either to autophagy activation or block of downstream steps of autophagy. Therefore, the simple determination of numbers of autophagosomes is insufficient for an overall estimation of autophagic activity. Accordingly, different methods have to be used together to ascertain increased autophagic activity. The execution of autophagy involves a set of autophagy-specific gene products, (Atg) that are evolutionarily conserved and play a key role in the formation of the

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Table 1
Routine methods for monitoring autophagy

Autophagy detection methods	Method description
Fluorescence microscopy	An imaging method to qualitatively detect fluorescently labeled autophagosomes
TEM or SEM	High-resolution imaging method to qualitatively observe autophagosome particles inside the cells at high magnification
Western blot	Traditional protein analysis method to quantify the total amount of a specific protein (such as LC3) in the target cell sample. The band compared to control will determine if authophagic activity exists
Flow cytometry	A sensible method to quantitatively detect fluorescently labeled autophagosomes or acidic particles inside the cells

autophagosomes [3]. Beclin-1, the mammalian orthologue of yeast Atg6, participates in autophagosome formation by interacting with human vacuolar protein sorting factor protein 34 (hVps34), but it [4] can be inhibited by the binding to the anti-apoptotic proteins Bcl-2 or Bcl-Xl [5]. Microtubule-associated protein light chain 3 (LC3), a mammalian homolog of yeast Atg8, is processed at its C terminus by Atg4 and becomes LC3-I, which resides in the cytosol. LC3-I can be subsequently conjugated with phosphatidylethanolamine (PE) to become LC3-II (LC3-PE) by a ubiquitination-like enzymatic reaction and associates with both the outer and inner membranes of the autophagosome. The methods that are currently used for the monitoring of autophagy are affected by numerous intrinsic pitfalls [6–10]. Therefore, the identification of LC3-II as a marker for autophagosomes [11] has greatly facilitated the detection of autophagy (through LC3-based biochemical and microscopic assays). The use of different methods in concert is required to accurately assess the autophagic process since there is no single "gold standard" for monitoring autophagy (Table 1) [12, 13]. Here, we will describe the flow cytometry methods for monitoring autophagy.

2 Materials

2.1 Common Materials

2.1.1 Disposables

1. 1.5 mL microcentrifuge tubes (Eppendorf, Hamburg, Germany). 100 × 20 culture dishes.
2. 15 and 50 mL conical centrifuge tubes. 175 cm^2 flasks for cell culture.
3. 6-, 24-well plates for cell culture.

2.1.2 Solutions

1. Growth medium for HeLa cells: Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, 4 mM l-glutamine, and 110 mg/L sodium pyruvate supplemented with 100 mM HEPES buffer and 10 % fetal bovine serum (FBS).
2. PBS (1×): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 in deionized water (dH_2O), adjust pH to 7.4 with 2 N NaOH.
3. Trypsin/ethylene diamine tetraacetic acid (EDTA): 0.25 % trypsin, 0.38 g/L (1 mM) EDTA4 Na in Hank's balanced salt solution (HBSS).
4. 20× Phosphate Buffered Saline (PBS): To prepare 1 L 1× PBS: add 50 mL 20× PBS to 950 mL dH_2O, mix.
5. 16 % Formaldehyde (methanol free).
6. 100 % methanol or 0.1 % (w/v) SDS in PBS.
7. Incubation Buffer: Dissolve 0.5 g Bovine Serum Albumin (BSA) in 100 mL 1× PBS. 10 % FBS in PBS. Store at 4 °C.
8. Secondary Antibodies: Anti-mouse, Anti-rabbit.
9. Fluorescent dyes used for the detection of autophagy.
10. Cytofluorimeter: C6 Accuri (Becton Dickinson, San Jose, CA) equipped with an argon ion laser emitting at 488 nm.

3 Methods

3.1 Monodansylcadaverine (MDC) Staining of Autophagic Vacuoles

1. Seed cells (25×10^5/well) in 6-well plates (*see* **Note 7**).
2. Following the desired stimuli for the induction or inhibition of autophagy, label cells with 50 μM MDC in growth medium (2 mL) for 15 min at 37 °C (Fig. 1).
3. Then, wash cells three times with PBS.
4. For flow cytometry analyses, trypsinize cells and combine them with floating cells from the medium. Resuspend cells in PBS containing 1 % FBS and analyze 10,000 cells using the FL-1 filter (*see* **Notes 1** and **2**). The percentage of positive cells should be assessed among a sample of statistical relevance (*see* **Note 3**) (Fig. 2).

3.2 Indirect Antibody Labeling of LC3-II

3.2.1 Fixation

1. Seed cells (25×10^5/wel) in 6-well plates.
2. Collect cells by centrifugation and aspirate supernatant.
3. Resuspend cells in 0.5–1 mL 1× PBS. Add formaldehyde to obtain a final concentration of 4 % (*see* **Note 4**).
4. Fix for 10 min at 37 °C.
5. For extracellular staining with antibodies that do not require permeabilization, proceed to immunostaining (*see* Subheading 3.2.3)

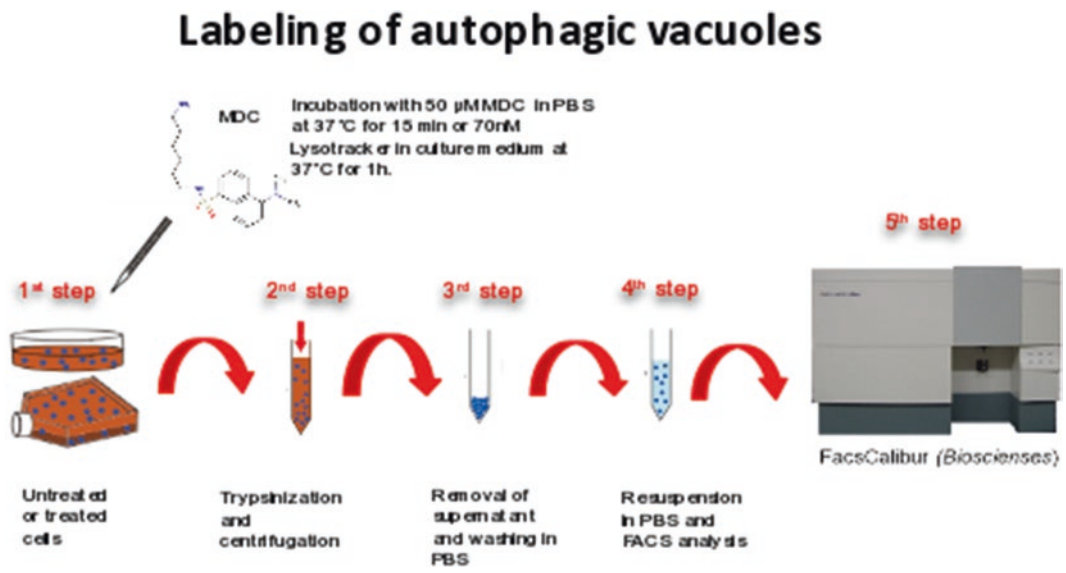

Fig. 1 Schematic representation of monodansylcadaverine (MDC) staining of autophagic vacuoles

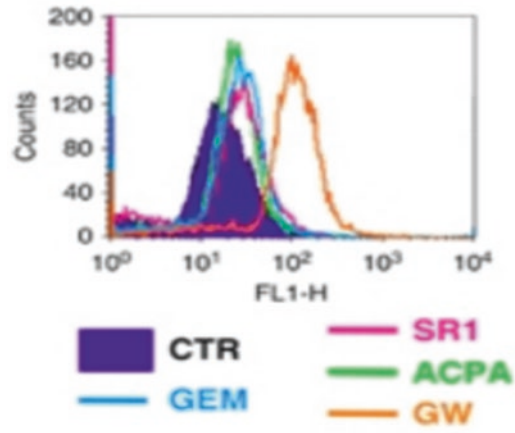

Fig. 2 Flow cytometry analysis of autophagic cells untreated (*CTR*) or treated with gemcitabine (*GEM*) or three different cannabinoids (SR1, ACPA, and GW) and stained with MDC

or store cells in PBS with 0.1 % sodium azide at 4 °C; for intracellular staining, proceed to permeabilization (*see* Subheading 3.2.2).

3.2.2 Permeabilization

1. Permeabilize cells by adding ice-cold 100 % methanol slowly to prechilled cells, while gently vortexing, to a final concentration of 90 % methanol. Alternatively, remove fix prior to permeabilization by centrifugation and resuspend in 90 % methanol as described above (*see* **Note 5**).

2. Incubate 30 min on ice.
3. Proceed with immunostaining (*see* Subheading 3.2.3) or store cells at −20 °C in 90 % methanol.

3.2.3 Immunostaining

1. Aliquot 0.5–1 × 10^6 cells into each assay tube (by volume) (*see* **Note 6**).
2. Add 2–3 mL incubation buffer to each tube and wash by centrifugation. Repeat.
3. Resuspend cells in 100 μL of primary antibody (prepared in incubation buffer at the recommended dilution). See individual antibody datasheet or product webpage for the appropriate dilutions.
4. Incubate for 1 h at room temperature.
5. Wash by centrifugation in 2–3 mL incubation buffer.
6. If using a fluorochrome-conjugated primary antibody, resuspend cells in 0.5 mL 1× PBS and analyze on flow cytometer; for unconjugated or biotinylated primary antibodies, proceed to immunostaining (**step 7**).
7. Resuspend cells in fluorochrome-conjugated secondary antibody or fluorochrome-conjugated avidin, diluted in incubation buffer at the recommended dilution.
8. Incubate for 30 min at room temperature.
9. Wash by centrifugation in 2–3 mL incubation buffer.
10. Resuspend cells in 0.5 mL PBS and analyze on flow cytometer by using the appropriate filter (*see* **Note 2**).

3.3 LC3-GFP Turnover

1. Seed cells (25×10^4/well) in 24-well plates (*see* **Note 7**).
2. 24 h later, transfect cells with a plasmid coding for the autophagosome marker LC3 fused with green fluorescence protein (GFP) [11], according to the following protocol. 4 μg of plasmid is diluted in 200 μL of Opti-MEM, at the same time as 5 μL of Lipofectamine™ is gently mixed with 200 μL Opti-MEM. After a first incubation of 5–10 min, the diluted plasmid solution and diluted Lipofectamine™ solution are gently mixed and incubated for another 20 min to promote the formation of Lipofectamine™:plasmid complexes (*see* **Note 8**).
3. Thereafter, add 30 μL of solution containing the Lipofectamine™:plasmid complexes to each well, in which the medium had been previously replaced with 500 μL of serum-free growth medium. Then incubate plates at 37 °C in 5 % CO_2 atmosphere for 4 h before adding 60 μL of FBS to restore the final FBS concentration of 10 % (as in complete growth medium) (*see* **Note 9**).

4. Cells are cultured for 24 h, or until they start to express the LC3-GFP fusion protein, prior to treatment with the desired stimuli (*see* **Note 10**).
5. At the end of stimulation, remove growth medium and wash cells twice with PBS.
6. Resuspend the cells in PBS containing 1 % FBS and analyze 10,000 cells by using the FL-1 filter (*see* **Notes 2** and **11**).

3.4 Lysotracker/LC3 Double Staining

1. Seed cells (25×10^5/well) in 6-well plates.
2. When cells have reached the desired confluence, remove the medium from the dish and add the pre-warmed (37 °C) medium containing Lysotracker probe at the final concentration of 50–75 nM (*see* **Notes 12–15**).
3. Incubate the cells for 30 min to 2 h under growth conditions appropriate for the particular cell type (*see* **Notes 16** and **17**).
4. Then, wash cells three times with PBS and immediately collect them by centrifugation.
5. Then, proceed with fixation/permeabilization (*see* Subheadings 3.2.1 and 3.2.2) and staining with antibody to LC3 (*see* Subheading 3.2.3).
6. For flow cytometry analyses, resuspend cells in PBS containing 1 % FBS and analyze 10,000 cells by using a dual FL1-FL2 filter combination (*see* **Notes 2** and **18–20**). The percentage of positive cells should be assessed among a sample of statistical relevance (*see* **Note 3**).

4 Notes

1. MDC is an autofluorescent molecule characterized by a relatively weak emission, peaking at 525 nm. However, when it interacts with membrane lipids (as occurring in AV) its emission shifts to 498 nm.
2. We routinely use a Becton Dickinson C6 Accuri cytofluorimeter, equipped with an argon ion laser emitting at 488 nm. The following channels are employed for the detection of fluorescent emissions: FL1 for MDC, FITC and GFP; FL2 for Lysotracker.
3. In order to perform each experiment with the appropriate controls, we used NF medium combined or not with an autophagy inhibitor that may provide positive and negative control conditions, respectively.
4. Prepare solutions with reverse osmosis deionized (RODI) or equivalent grade water.

5. Account for isotype matched controls for monoclonal antibodies or species matched IgG for polyclonal antibodies.
6. Excessive confluence (at levels coinciding with a reduction of proliferation) should be carefully avoided, since it results in a significant decrease of the transfection efficacy. In particular, we found that optimal transfection rates are attained when cells are slightly more confluent (70 %).
7. When the diluted Lipofectamine™ and plasmid solution are mixed, the solution may appear cloudy. The liposome-mediated plasmid transfection protocol is carried out entirely at RT under a common safety cabinet. However, it is recommendable to keep the tubes containing the stock solutions of Lipofectamine™ and plasmid in an ice bath (and to return them to storage conditions immediately after use), to avoid the degradation of reagents, and to minimize solvent evaporation (both of which may eventually affect the concentration of the stocks).
8. Transfection complexes should be added to cells dropwise because of their very high affinity for the plasma membrane, to cover the whole surface of the growth medium and to avoid intrawell variations of the transfection efficiency.
9. Seeding concentration depends on the duration and strength of the subsequent treatments. As a guideline, for treatments of 24 h or less (administered 24 h after plating) we use to seed 25×10^5 cells (6-well plates).
10. An adequate control has to be carried along the entire experimental procedure of transfection and treated as samples. To this aim, untransfected cells or cells treated only with lipofectamine may be used. But a more stringent control is represented by cells transfected with the empty cloning vector.
11. Lysotracker probes are fluorescent acidotropic, lysosomotropic, readily cell-permeant fluorochromes for labeling acidic compartments in living cells. These molecules accumulate in acidic subcellular compartments including autophagolysosomes. Since such probes alone are not specific for assessing autophagy, they can be used in combination with LC3-II to quantify autophagy.
12. Before opening, allow the vial to warm to room temperature and then briefly centrifuge the vial in a microcentrifuge to deposit the DMSO solution at the bottom of the vial.
13. The concentration of probe for optimal staining will vary depending on the application.
14. For the Lysotracker® probes, we recommend working concentrations of 50–75 nM. To reduce potential artifacts from overloading, the concentration of dye should be kept as low as possible.

15. The staining conditions may need to be modified depending upon the particular cell type and the permeability of the cells or tissues to the probe, among other factors.
16. If the cells are incubated in dye-free medium after staining, we often observe a decrease in fluorescent signal and cell blebbing.
17. If the cells do not appear to be sufficiently stained, we recommend either increasing the labeling concentration or increasing the time allowed for the dye to accumulate in the lysosomes.
18. In order to avoid probe-dependent toxicity to the cells, cytofluorimetric acquisitions should be performed within 30 min.
19. When large series of samples are to be analyzed (>12 tubes), the interval between labeling and cytofluorimetric analysis should be kept constant.
20. It is important to use the appropriate combination of Lysotracker probes and fluorescent antibodies directed against LC3. Lysotracker probes exist in several variants, which exhibit distinct excitation and emission spectra, to facilitate double or triple stainings. For instance, we use Lysotracker Red and label AV with FITC-conjugated antibodies (emitting in green).

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Chapter 17

Echocardiography-Guided Intramyocardial Injection Method in a Murine Model

Kay Maeda, Rick Seymour, Marc Ruel, and Erik J. Suuronen

Abstract

Cardiac regenerative therapy has received attention as a potentially revolutionary approach for treating the damaged heart. The mouse model of myocardial infarction (MI) remains one of the most common tools for the evaluation of such new therapies. Typically, intramyocardial administration of cells or biomaterials in mice is performed by an open-chest surgical procedure, but less invasive delivery methods are becoming available. Echocardiography-based transthoracic myocardial injection is one such minimally invasive approach that can reliably deliver therapeutics to the target site with limited complications and quick recovery for the animal following the procedure. Here, we will describe the method of echocardiography-guided intramyocardial injection in a mouse MI model.

Key words Intramyocardial injection, Echocardiography, Myocardial infarction, Stem/progenitor cells, Cardiac tissue engineering

1 Introduction

Over the last decade, many strategies using stem/progenitor cell and other therapeutic agents such as growth factors, drugs, and biomaterials have been investigated to regenerate and restore function to the damaged myocardium [1–5]. In their preclinical development, these therapies are typically tested in mouse models of myocardial infarction (MI), which are important for establishing efficacy and providing insight into the underlying mechanism(s) of the treatment [6]. The MI model is established by ligation of the left anterior descending coronary artery via an invasive open-chest surgical procedure, after which the therapeutic is administered at a predetermined time-point post-MI. Direct intramyocardial injection is often the most effective route of administration for these therapeutics [4, 7–10]. This mode of delivery has typically required a second open-chest procedure, which can increase mortality rates, the operating time, and the cost of the experiments. In recent years, minimally invasive techniques for establishing mouse models

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and testing novel therapies (in animal models and in the clinic) have emerged that alleviate the need for the open-chest procedure [6, 11–14]. Here, we describe a technique for echocardiography-guided intramyocardial injection that has been used for the minimally invasive and effective delivery of cells and injectable hydrogels in an experimental mouse model of MI [15–17].

2 Materials

2.1 Ultrasound Imaging System Setup

1. VisualSonics Vevo770® high-resolution imaging system.
2. RMV707B high frame rate scanhead.
3. Stage heater.
4. Heart and temperature monitor.

2.2 Pretreatment for Mice

1. Buprenorphine (0.05 mg/kg of body weight).
2. Anesthetic isoflurane vaporizer (Datex-Ohmeda Isotec 5 vaporizer).
3. Supply gas (oxygen).
4. Hair removal cream.
5. Lubricant eye ointment (Tears Natural P.M.; Alcon).

2.3 Functional Assessment

1. Anesthetic isoflurane vaporizer.
2. Supply gas (oxygen).
3. Redux® Creme electrolyte cream.
4. Aquasonic® ultrasound transmission gel.

2.4 Injection

1. Injectate (e.g., cells, injectable biomaterial): maximum of 10–15 μL per injection site, up to total of 50 μL per mouse heart in up to five injection sites (*see* **Note 1**).
2. Sterile Terumo U100 insulin syringe and needle (1 mL; 27G × ½) (*see* **Note 2**).

3 Methods

All the procedures were performed in accordance with the Canadian Council for Animal Care Guidelines for the Care and Use of Laboratory Animals and with the approval of the University of Ottawa Animal Care Committee. The following method is for the echocardiography-guided delivery of therapeutics to the infarcted mouse heart. The myocardial infarction procedure itself is not described here.

3.1 Surgical and Imaging System Setup

3.1.1 Preparation of Surgical Area and Supplies

1. Ultraviolet (UV) irradiation treatment is required for all surgical equipment (e.g., stage, scanhead) before the procedure.
2. All of the surgical instruments (e.g., needles, forceps) and supplies should be sterilized.
3. The surgeon should wear a facemask, hair bonnet, sterile surgical gown, and sterile gloves.

3.1.2 Ultrasound Imaging System Setup (Fig. 1)

1. Warm the stage up to 37 °C to maintain mouse body temperature.
2. Turn on the Heart/Temperature monitor.
3. Place the 707B scanhead probe in the probe holder, positioned such that the probe will provide a sagittal plane image.

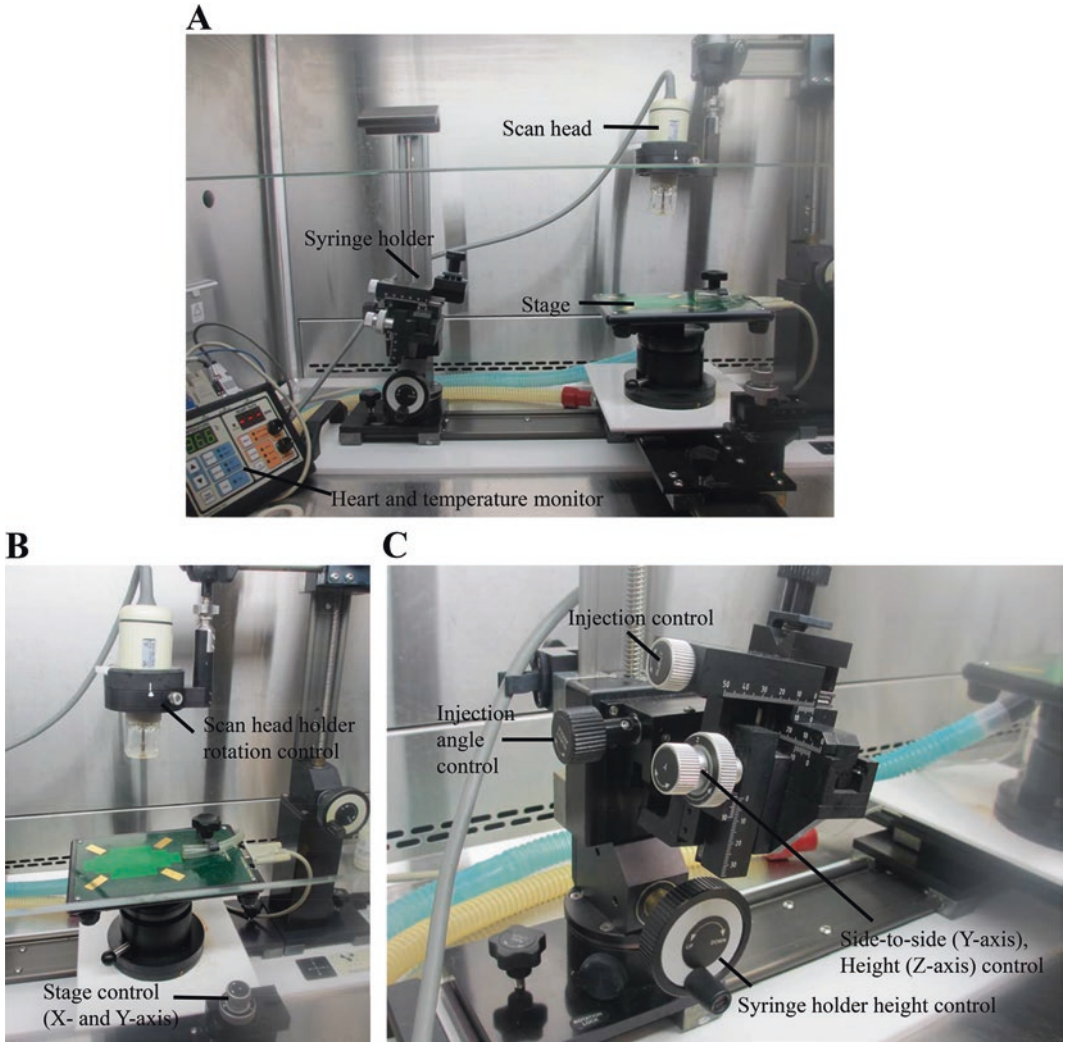

Fig. 1 Ultrasound imaging system setup. (**a**) Overall view of echocardiography-guided injection system. (**b**) Scanhead and stage controls. (**c**) Injection controls

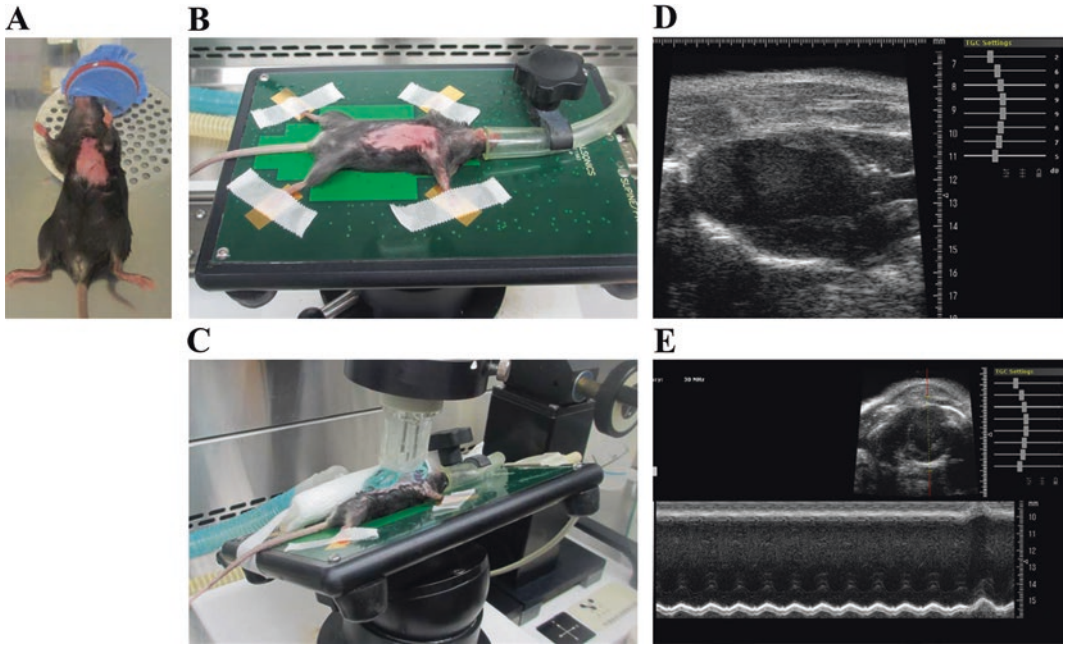

Fig. 2 Mouse positioning and representative echocardiography images. (**a**) Chest hair shaving. (**b**) Mouse setting on the stage. (**c**) Orientation of the animal stage and transducer to obtain the optimal long axis image of the heart. (**d**) Long axis image from the aortic ostium to the apex. (**e**) Short axis image and M-mode image of the left ventricle at the level of the midpapillary muscles

3.2 Pretreatment of Mice

1. Buprenorphine (0.5 mg/kg of body weight, subcutaneous) is administered to the mice prior to initiating the intramyocardial injections.
2. Place the mouse into an induction chamber with 2 % isoflurane until anesthetized.
3. Place the anesthetized mouse onto the warmed platform in the supine position and maintain 2 % isoflurane through a nose cone during the entire procedure (*see* **Note 3**).
4. Apply lubricant eye ointment onto both eyes to prevent eyes from drying out. Shear the chest hair with hair removal cream so that the chest is fully exposed. (Fig. 2a).
5. Apply conductive electrolyte cream to the paws and tape each paw to the corresponding electrode foot pads on the stage for electrocardiogram (ECG) measurement (Fig. 2b) (*see* **Note 4**).
6. Apply sufficient ultrasound transmission gel on the mouse's chest (*see* **Note 5**).

3.3 Visualization of the Mouse Heart

1. To obtain the optimal cardiac longitudinal image, the stage is rotated to orient the mouse in the right half lateral position.
2. Advance the ultrasound transducer down into the transmission gel and visualize the longitudinal heart in B-mode so that aortic valves are well-visualized (*see* **Notes 6** and **7**).

3. Using the scanhead holder rotation control, visualize the long axis (from aortic ostium to the apex) of the heart (Fig. 2c, d) (*see* **Note 8**).
4. Optional: Store the ECG-based Kilohertz Visualization (EKV) reconstruction image for follow-up analysis (*see* **Note 9**). Also, an M-mode image of the short-axis (obtained by a 90° counter-clockwise rotation of the scanhead) may be necessary to characterize wall motion of the infarcted myocardium (Fig. 2e) (*see* **Note 10**).

3.4 Injection of Therapeutic

1. Direct the scanhead parallel to the axis of the syringe/needle holder.
2. Incline the stage such that the mouse posterior is positioned downward (Fig. 3). Keep the image on your target injection site in the longitudinal view by using the stage controls (*see* **Note 11**).
3. Set the syringe, which has been preloaded with the injectate, into the syringe holder (*see* **Note 12**).
4. Advance the needle toward the chest until the image of the needle appears on the screen. Minor side-to-side or height adjustments of the needle using the stage controls may be necessary to obtain a clear image of the needle.
5. Slowly advance the needle through the skin, intercostal, and into the target myocardium by using the injection control knob (Fig. 4a–d) (*see* **Note 13**). Make sure that the whole needle bevel is observed within the myocardium (*see* **Note 14**).

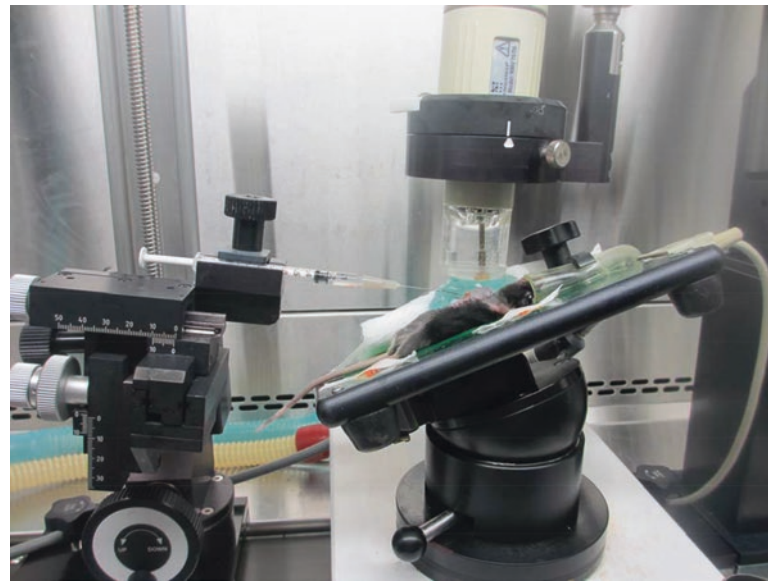

Fig. 3 Optimal mouse positioning for echocardiography-guided injection in the longitudinal imaging plane

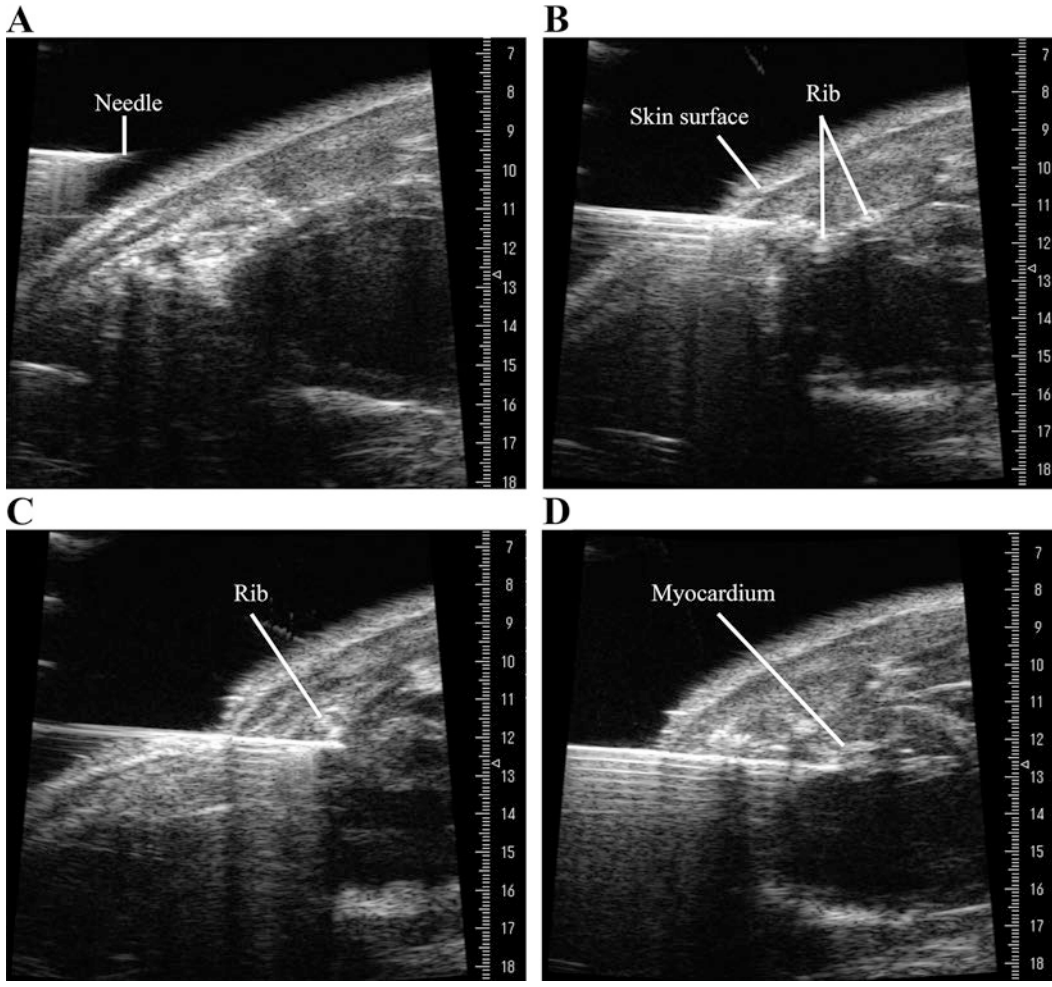

Fig. 4 Representative images showing intramyocardial injection. The 27 G needle is moved (**a**) toward the chest skin surface, (**b**) through the skin and (**c**) intercostal space, and (**d**) into the myocardium

6. Inject a very small amount of injectate per injection site (10–15 μL for the mouse heart; *see* **Note 15**). A larger injectate volume may cause damage or rupture of the ventricular wall.
7. Once the injection is complete, withdraw the needle straight out quickly.
8. To inject serially at multiple sites, once the needle is withdrawn from the myocardium completely, advance it into another site after shifting the needle sideways by using the side-to-side control knob (*see* **Note 16**).
9. Following the injection(s), place the mouse into its own cage and observe until the mouse is fully recovered from anesthesia and can move around the cage normally.

4 Notes

1. Injectates are typically delivered to 1–5 sites in the myocardial scar and border zone (identified in the cardiac image by the thinned myocardial wall and reduced wall motion).
2. When cells are injected, 26–30 G needles are preferable but this will depend on the cell size [18, 19].
3. Keeping normothermia provides normal homeostasis in mice. Hypothermia causes bradycardia, ventricular dilatation, or possible organ damage.
4. Appropriate heart rate is between 350 and 400 beats per minute during the procedure.
5. Avoid making air pockets in the ultrasound transmission gel between the transducer and the skin surface. Air bubbles disturb the ultrasound image. If bubbles occur, remove the gel and reapply without air bubbles.
6. Adjust the aortic ostium to visualize it at the triangular arrow of the depth ruler that indicates the focal length of the transducer.
7. To adjust gain of the image quality in targeted areas (depths), drag the time-gain compensation sliders. Sliders can be dragged left or right to decrease/increase the receive gain of the B-mode image.
8. Additional counter-clockwise rotation of the scanhead holder control may be required to acquire the long axis view from the aortic ostium to the apex.
9. The EKV can reconstruct representative cardiac cycles that can be used to calculate different cardiac function parameters such as ejection fraction, fractional area change, stroke volume, and cardiac output.
10. The mid-papillary is the landmark to obtain a reproducible short axis image for M-mode image acquisition.
11. Minor X- and Y-axis adjustment of stage controls are needed to keep the view of the target site.
12. If you inject cells, mix cells well right before injection to ensure even distribution within the mixture.
13. Advance the needle in the horizontal direction. If it is difficult to go through the skin, incline the needle with respect to the vertical direction to the skin surface to help the needle pierce through.
14. Pull the syringe plunger and confirm that blood is not drawn from the left ventricle chamber. This ensures that the injectate will not be administered or leak into the chamber.

15. During the injection, the injected site can be visible as hyperechoic region.
16. Injectate can be delivered up to a total of 50 μL per mouse heart.

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Chapter 18

Noninvasive Assessment of Cell Fate and Biology in Transplanted Mesenchymal Stem Cells

Federico Franchi and Martin Rodriguez-Porcel

Abstract

Recently, molecular imaging has become a *conditio sine qua non* for cell-based regenerative medicine. Developments in molecular imaging techniques, such as reporter gene technology, have increasingly enabled the noninvasive assessment of the fate and biology of cells after cardiovascular applications. In this context, bioluminescence imaging is the most commonly used imaging modality in small animal models of preclinical studies. Here, we present a detailed protocol of a reporter gene imaging approach for monitoring the viability and biology of Mesenchymal Stem Cells transplanted in a mouse model of myocardial ischemia reperfusion injury.

Key words Stem cells, Molecular imaging, Reporter gene, Bioluminescence, Luciferase, Tracking, Monitoring, Heart

1 Introduction

In the first decade of the twenty first century we have been pelted with all sort of cell-based therapies in clinical cardiovascular practice [1–4] stimulated to a good degree by the promising results of some preclinical studies [5–8]. A wide range of adult stem cell types have been used, including Mesenchymal Stromal Cells (MSCs) [9, 10], Bone Marrow-derived Mononuclear Cells (BMMNCs) [4], and Hematopoietic Stem Cells (HSCs) [11, 12]. However, the bottom line has been a deflated enthusiasm due to insufficient retention and engraftment of the cells, which may limit their regenerative capacity [13–15]. Thus, the need to further investigate in vivo the kinetics of cell integration into the host tissue (both in preclinical and clinical settings) has become critical for the advancement of regenerative medicine. Until recently, assessment of the fate of transplanted stem cells has relied on traditional ex vivo assays and molecular techniques (i.e., histology, western blotting) [16]. Although these methods are easy to carry out for the molecular biologists and do not require special instruments, they involve invasive procedures and are limited

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in their capacity to monitor temporal changes in the living subject. This has inspired the development of novel research strategies devoted to improving the delivery as well as the biology of transplanted stem cells. Within this context, the advent of molecular imaging represents a far-reaching milestone for the noninvasive monitoring of cell fate in vivo [17–20].

Direct (fluorophores [21], superparamagnetic iron oxide particles [22], radioisotopes [23, 24]) and indirect (reporter genes) labeling [16, 25] of stem cells may be used to assess their short and long-term distribution, along with their viability, proliferation, and functional interaction with the host microenvironment.

When choosing the appropriate technique for any experimental design, it is critical to keep in mind the biological and biochemical properties as well as the sensitivity of each strategy. While direct labeling is used to monitor cell fate only shortly after transplantation, mainly due to the progressive dilution of the signal—as a consequence of cell division—and cell toxicity issues—which may vary depending on the agent and doses used, reporter gene imaging allows long-term assessment of cell fate with longitudinal and repetitive imaging [26, 27]. Using this strategy, cells are engineered to over-express or produce *de novo* an enzyme, receptor, or protein: when this protein interacts with an exogenously administered substrate, it results in a signal that can be used to distinguish not only implanted cells from endogenous cells with high specificity, but also modulations in intracellular functions [28]. However, it is critical to keep in mind that the manipulation of DNA sequences may alter the biology of transplanted stem cells. Furthermore, transgene expression is a very complex process that involves many molecular steps: this may limit the amount of protein produced as well as the strength of the signal.

During the last ten years, using reporter gene strategies designed to express the firefly luciferase (Fluc) or renilla luciferase (Rluc) gene, Bioluminescence Imaging (BLI) has been successfully adopted to monitor in vivo stem cell viability and engraftment [18, 29, 30] as well as to study transplanted stem cell biology and its interaction with the microenvironment [28] (Fig. 1). Due to the lack of spatial resolution and tissue depth penetration, the use of BLI is restricted to small animal studies (rats and mice) [26]. However, this strategy can be adapted to clinically used imaging modalities, such as Positron Emission Tomography (PET) or Single-Photon Emission Computed Tomography (SPECT), using, for instance, thymidine kinase (TK) or human sodium-iodide symporter (hNIS) as reporter genes, respectively [19, 27]. Therefore, BLI may be considered a starting step in the development of novel imaging strategies in high-throughput preclinical studies.

Here, we describe in detail the materials and methods necessary to perform in vivo and ex vivo BLI of MSCs transplanted to the myocardium of a mouse model of ischemia/reperfusion injury.

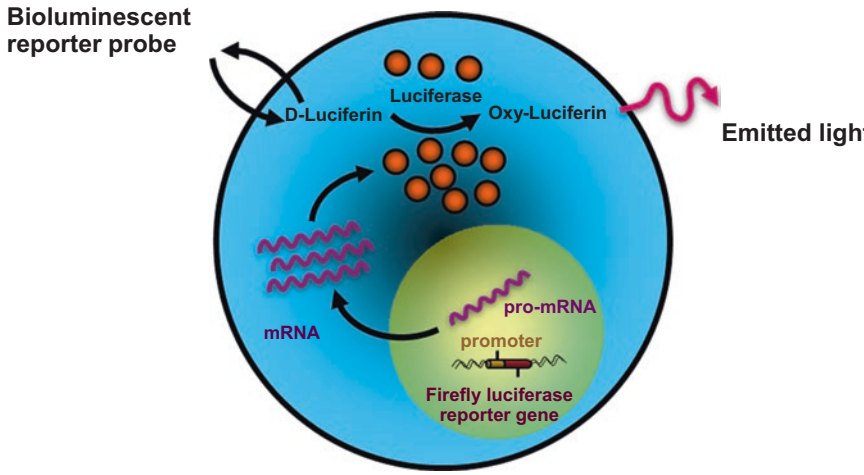

Fig. 1 Reporter gene technology for bioluminescence imaging. D-Luciferin, a light-emitting compound, is the substrate of the firefly luciferase reporter gene. In the presence of oxygen, adenosine triphosphate (ATP), and magnesium, the enzyme luciferase catalyzes the oxidation of D-Luciferin to form oxyluciferin in an electronically excited state. The reaction releases a photon of light as oxyluciferin returns to the ground state. These photons can be detected, collected, and quantified by a CCD camera

2 Materials

Prepare and store all reagents at 4 °C or on ice, unless stated otherwise. Prepare at the same time all the necessary controls. Carefully follow all waste disposal regulations for waste materials.

2.1 Cell Culture Components

1. Culture media: Dulbecco's Modified Eagle Medium (DMEM), 10 % Fetal Bovine Serum (FBS), 2 mM L-Glutamine, 1 % Penicillin/Streptomycin.
2. Trypsin: trypsin-EDTA (0.25 %).
3. Buffered solution: Phosphate Buffered Saline (PBS), pH7.4.
4. Tissue culture flasks: T25, T75, or T175 flasks, 24-well plates.
5. CO_2 incubator for cell culture at 37 °C and 5 % CO_2.

2.2 Reporter Gene Technology

2.2.1 Transient Transfection (Plasmid Vectors)

1. Early-passage MSCs (*see* **Note 1**).
2. Effectene Transfection Reagent (Qiagen): Buffer EC (15 mL), Enhancer (1 mg/mL, 0.8 mL), Effectene Transfection Reagent (1 mg/mL, 1 mL). Store at 4 °C.
3. Plasmid vectors: CMV-Fluc or CMV-Rluc for monitoring of cell viability and engraftment; X-Fluc (X = pathway-specific promoter) for monitoring of cellular functions; Null-Fluc (promoter-less vector) as a control vector.

2.2.2 Stable Infection (Retroviral Vectors)

1. Early-passage MSCs (*see* **Note 1**).
2. Retroviral vectors: CMV-Fluc or CMV-Rluc for monitoring of cell viability and engraftment; X-Fluc (X = pathway-specific promoter) for monitoring of cellular functions; Null-Fluc (promoter-less vector) as a control vector (*see* **Note 2**).
3. Polybrene Infection/Transfection Reagent. Store at −20 °C (*see* **Note 3**).
4. Antibiotic selection: Geneticin Selective Antibiotic (G418 Sulfate, Gibco) could be used as selective antibiotic in the concentration range of 200–500 μg/mL for most mammalian cells in case the retroviral vector has the correspondent resistance gene (*see* **Note 4**).

2.3 Surgical Materials

1. Surgical tools (Fine Science): Delicate Forceps (Smooth/Angled 45°/9 cm) and Bonn-Strabismus Scissors (Straight/Blunt-Blunt/9 cm) for skin and muscle, Cohan-Vannas Spring Scissors (Curved/Sharp/5.7 cm/6 mm Cutting Edge) for ribs interspace, Chest retractor, Moria MC31 Iris Forceps (Serrated/Curved/10 cm), and Moria MC31/B Iris Forceps (Smooth/Curved/10 cm) for pericardium removal, Castroviejo Micro Needle Holders (Curved/9 cm/with Lock), and two Suture Tying Forceps (10 cm) for left anterior descending coronary artery (LAD) ligation, Halsey Micro Needle Holder (Tungsten Carbide/Straight/Serrated/12.5 cm/with Lock) for suturing muscle and skin (*see* **Note 5**).
2. Glass beads sterilizer.
3. Heating pad for animal surgery and animal recovery (*see* **Note 6**).
4. Anesthetic: Isoflurane.
5. Lactated ringers for fluids replacement.
6. Analgesic: Buprenorphine.
7. Puralube Vet Ointment.
8. Preoperative hair removal: depilatory cream.
9. Endotracheal tube (20-gauge, ¼”).
10. Intubation panel.
11. Small animals laryngoscope.
12. Physio Suite with MouseVent Automatic Ventilator module and MouseSTAT Pulse Oximeter/ Heart Rate module (Kent Scientific).
13. Surgical microscope (Stereo Microscope Leica M125, Leica Microsystems).
14. Povidone Iodine applicators and Ethanol pads.
15. Sutures: 9-0 Ethilon suture, 6-0 Silk suture, and 6-0 Vicryl suture.

16. UltiCare Insulin Syringe U-100, 30-GAUGE × ½".
17. Antibiotic treatment: Triple Antibiotic Ointment.
18. Cotton Swabs and gauzes.

2.4 Bioluminescence Imaging Technique

1. D-Luciferin Firefly, Potassium Salt: dissolve D-Luciferin in PBS without calcium and magnesium to a final concentration of 10 mg/mL. Filter sterilize the solution through a 0.2 μm filter. Store at −20 °C (*see* **Note 7**).
2. 29-gauge insulin syringe.
3. Anesthetic: Isoflurane.
4. Cooled charge-coupled device camera (Xenogen).

2.5 Luminometry Components

1. 5× Passive Lysis Buffer (Promega, store at −20 °C): dilute in H_2O and keep on ice.
2. Tissue homogenizer (IKA RW20 digital).
3. Luciferase Assay Reagent (substrate of Fluc, Promega). Aliquot and store at −80 °C (*see* **Note 7**).
4. Coelenterazine (Biotium, 1 mg): dissolve in 1 ml Ethanol to prepare a stock solution of 1 μg/μL. Aliquot 13 μL per tube into black microtubes to shield from light. Store at −80 °C (*see* **Note 7**). Before use, add 1.3 mL of PBS and vortex. Keep on ice.
5. Luminometer (Turner Designs 20/20).

3 Methods

3.1 Reporter Gene Labeling of Mesenchymal Stem Cells

3.1.1 For Plasmid Transfection

1. Plate MSCs at a density of 1×10^4 cells per cm^2 18 h before the transfection in T25 culture flasks.
2. Following manufacturer's instructions mix plasmid DNA (2.5–3 μg) with Buffer EC and Enhancer (The ratio of DNA to Enhancer is 1 μg DNA to 8 μL Enhancer). Vortex briefly and incubate for 5 min at room temperature (*see* **Note 8**).
3. Add Effectene Reagent (in our hands, for MSCs the ratio of DNA to Effectene is 1 μg DNA to 10 μL Effectene. This should be optimized for every new cell line and DNA construct used). Vortex and incubate for 10 min at room temperature.
4. Add culture media and mix by pipetting up and down several times.
5. Add the transfection cocktail onto the cells and incubate for 6 h at 37 °C.
6. Wash the cells once with PBS and add fresh culture media.
7. After 24 h incubation at 37 °C, prepare the cells for the injection.

3.1.2 For Retroviral Infection

1. Plate MSCs at a density of 1×10^4 cells per cm^2 24 h before the infection in a 24-well plate.
2. Change media and infect MSCs with a multiplicity of infection (MOI) of 10 in the presence of Polybrene Infection/Transfection Reagent (8 μg/ml).
3. Incubate the cells overnight at 37 °C.
4. Change the media every day. When cells reach 70 % confluency, split them 1:5 into selection media (according to the resistance gene of the viral vector).
5. Prepare the cells for the injection.

3.2 Surgical Protocol for Induction of Ischemia/Reperfusion Injury and Cell Delivery

1. The day before the surgical procedure, shave the chest area of the mouse and depilate with depilatory cream. This will minimize the stress the day of the surgery.
2. Before starting the surgery, clean the working area, surgical tools, and accessories. Turn on the instrument sterilizer, the heating pad so that the overlying surgery panel can warm up, and the heating plate with recovery cage on top of it.
3. Turn on the anesthesia system, with inflow to the induction chamber open, at 4 % isoflurane and a flow rate of approximately 2 L/min.
4. Place the mouse in the induction chamber. When the mouse becomes unconscious (within 1–2 min), weigh and mark it.
5. Open the flow to the ventilation cone. Place the mouse on the intubation panel and place cone near mouse's face to keep anesthetized during intubation.
6. Inject 1 mL of Lactated ringers subcutaneously in the back to keep the animal hydrated.
7. Inject buprenorphine (0.1 mg/kg body weight) subcutaneously in the shoulder.
8. Put a dab of ointment (Puralube Vet Ointment) directly on the eyes to prevent drying during surgery.
9. Intubation procedure: place the mouse in a supine position, fixed at its front teeth by a piece of suture, and tape its tail. With the laryngoscope visualize the trachea. Insert the endotracheal tube between the two vocal cords (*see* **Note 9**).
10. Connect the tube to the ventilation machine. Input the animal weight: the machine will calculate the optimal respiration rate and tidal volume. Reduce flow rate to 1 L/min. Secure the ventilation tubing on the surgery panel with a tape.
11. Once the animal is breathing with the ventilator, keep isoflurane at 1.5–1.8 %.
12. To position the animal suitably for the surgery, fix both forelimbs to the right side of the animal; fix the right hind leg in

parallel with the tail and the left hind leg turned to the right side. The orientation from the surgeon's viewpoint is horizontal, tail right, head left.

13. Cover the shaved area with Povidone-Iodine using a cotton tip applicator. Clean with alcohol.
14. Focus the microscope on the left side of the chest area.
15. With blunt scissors and forceps, make a 1.5 cm long skin incision over the left thorax area, just 1 mm below the first nipple visible from the left axilla.
16. Loosen the skin from the connective tissue/muscle layers by blunt dissection (prodding the scissors under the skin and opening them).
17. Make an incision of 1 cm in the muscles between the fourth and the fifth intercostal space. Using Cohan-Vannas Spring Scissors, perforate and cut the intercostal muscle layer 1 mm away from the sternum (*see* **Note 10**).
18. Retract the chest.
19. Zoom in on the heart. Open the pericardium using two pairs of small rounded forceps. Expose the upper and middle parts of the left ventricle with its partly overlying auricle (atrium) and blood vessels.
20. Localize the LAD (*see* **Note 11**) (Fig. 2).
21. Ligate the LAD distal to the left auricle, 2 mm below the atrium edge (*see* **Note 12**): hold a 9-0 ethilon suture with a small needle holder and insert it shallowly into the myocardium, enclosing the LAD and approximately four times its diameter of surrounding myocardium (*see* **Note 13**). Tightly compress the LAD in its middle third by a thin piece of plastic tubing secured by the suture. Confirm ischemia by the appearance of pallor over the anteroapical LV myocardium, along with hypokinesis/akinesis (Fig. 2) (*see* **Note 14**).
22. After 35 min of ischemia, cut the suture and remove the tubing to allow reperfusion (Fig. 2).
23. After about 10 min of reperfusion, using a 30-gauge insulin syringe, inject cells into the myocardial wall. Inject around 4×10^5 cells in 30 μl of PBS (2 spots of 15 μL each) in the peri-infarct area (Fig. 2).
24. Remove the retractor.
25. Decrease anesthesia to 1–1.2 % isoflurane.
26. Insert the chest tube (24-gauge) through the skin, the muscles, and between the third and the fourth intercostal space.
27. Close the thoracic wall in three layers: first the ribs with individual 6-0 silk sutures, then the muscles with running 6-0 vicryl suture, and, finally, close the skin with running 6-0 vicryl sutures.

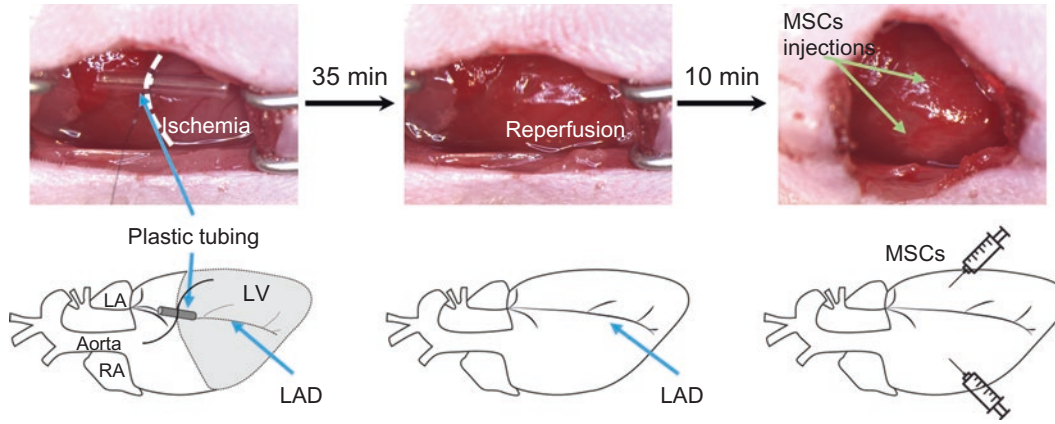

Fig. 2 Ischemia/reperfusion injury and cell delivery. Schematic representation of the surgical procedure for the induction of an ischemia/reperfusion injury in mice followed by intra-myocardial transplantation of Mesenchymal Stem Cells. LA: left atrium, RA: right atrium, LV: left ventricle, LAD: left anterior descending coronary artery, MSCs: Mesenchymal Stem Cells

28. Aspirate air from the chest with a 1 mL tuberculin syringe and remove the chest tube.
29. Clean the incision area. Apply the triple antibiotic ointment to the incision area.
30. Turn off the isoflurane and allow the animal to recover increasing the flow rate (oxygen only) to 2 L/min. The animal should be able to recover in less than 1 min (*see* **Note 15**).
31. Extubate the animal as soon as it begins fighting the tube and breathing on its own.
32. Place the animal into a clean cage on the heating pad and monitor frequently for any sign of discomfort.
33. Administer buprenorphine (0.1 mg/kg body weight) subcutaneously every 8–12 h for at least 3 days postsurgery.

3.3 In Vivo Bioluminescence Imaging of Transplanted Mesenchymal Stem Cells

1. Shave the chest area of the mouse and depilate with depilatory cream (*see* **Note 16**).
2. Turn on the anesthesia system, with inflow to the induction chamber open, at 4 % isoflurane and a flow rate of approximately 2 L/min.
3. Place the mouse in the induction chamber.
4. When the mouse becomes unconscious, inject intraperitoneally100 μL of the reporter substrate D-Luciferin Firefly, using a 29-gauge insulin syringe.
5. Place the animal in the cooled charge-coupled device camera (Fig. 3) in a supine position. During imaging procedure keep isoflurane at 1–1.5 % (*see* **Note 17**).

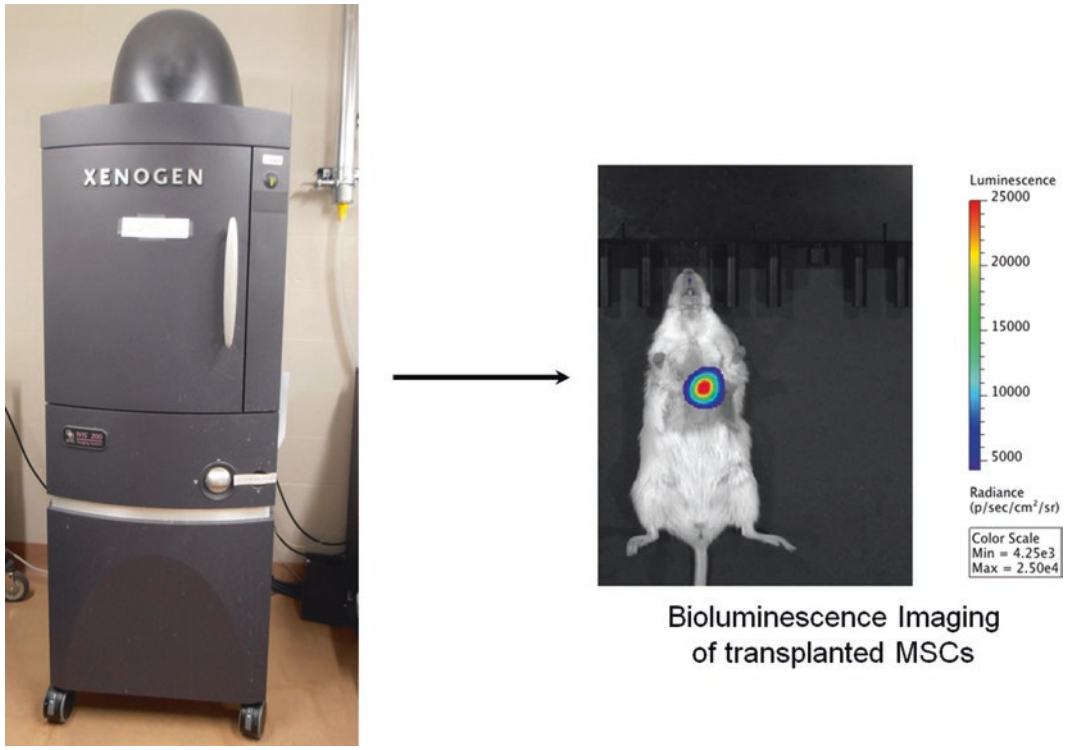

Fig. 3 Bioluminescence Imaging of MSCs transplanted to the myocardium. Representative image of bioluminescent imaging-based reporter gene strategy. Cells that carry the reporter gene firefly luciferase were delivered to the myocardium of a mouse that underwent an ischemia/reperfusion injury. The animal was placed supine in a charge-coupled device camera, after substrate (D-Luciferin) administration. Color images of visible light are superimposed on photographic images of animals with a scale in photons per second per square centimeter per steradian (sr)

6. Image animal for 20 min using one-minute high-sensitivity, acquisition scans (Fig. 3).
7. Quantify bioluminescence as total radiance (photons/s/cm^2/sr) over the area of the heart, using a region of interest kept at constant size for all scans (*see* **Note 18**).
8. After the imaging session is complete, let the animal recover in a cage placed on a warm pad (*see* **Note 19**).

3.4 Ex Vivo Luminometry of Heart Homogenates

1. Sacrifice the animal with an overdose of CO_2.
2. Immediately harvest and weigh the organs.
3. Wash twice with PBS.
4. Add lysis buffer (4 mL/g harvested tissue).
5. Homogenize for 30 s.
6. Centrifuge at 21,000 × *g* for 15 min at 4 °C.
7. Assay 20 μL supernatant with 100 μL substrate (Luciferase Assay Reagent or Coelenterazine) on luminometer. Be sure to vortex each sample before assaying.

4 Notes

1. Young cells are required for better efficiency of transfection.
2. Recently, novel strategies have been developed for stable genomic integration to facilitate targeted editing of the genome by creating double-strand breaks in DNA at user-specified locations. These technologies use DNA-binding proteins such as Zinc Finger Nucleases (ZFN), Transcription activator-like effector nucleases (TALEN), or Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR)/Cas9 systems [31].
3. The efficiency of retroviral infection is enhanced significantly in MSCs, by including polybrene during the infection.
4. This step allows the positive selection of those cells that were efficiently infected by the retroviral vector. An alternative could be Puromycin (1–5 μg/mL).
5. Autoclave all instruments before surgical session. Sterilize instruments in between animals using a glass beads sterilizer to avoid infections.
6. Do not overheat the pad as it may cause burns to the animal.
7. The reagent is light sensitive. Minimize exposure to light.
8. The enhancer condenses the DNA molecules and the buffer provides optimal salt conditions for efficient DNA condensation.
9. The mouse should be deeply anesthetized to avoid pharyngeal reflex (gag reflex), which makes the intubation procedure more difficult. Insert the endotracheal tube only if the trachea and the vocal cords are clearly visible. Do not try to force entry, but change the position of the tube tip. The tube tip is angled and should face upward. Do not perform more than three attempts as the trachea may be damaged irreversibly.
10. Choose the intercostal space for thoracotomy based on curvature, after first rib that is less curved than the rib above. Be careful avoiding contact with the lungs.
11. This requires some expertise. If necessary, carefully lift the atrium. The left anterior descending coronary artery is bright red to orange/pink, as opposed to the veins, which are dark red, is pulsatile, and runs from below the left auricle to the apex.
12. If the heart rate is too high, temporarily increase the anesthesia to 2.5 % isoflurane.
13. Avoid entering the LV cavity, but go deep enough to see the LAD pulsate over the needle.
14. For a murine model of myocardial infarction perform a permanent ligation of the LAD: close the suture using a double surgeon's knot, fixed with two extra half hitches. The heart

region below the knot should become pale in few seconds. The knot is not released.

15. The surgical procedure should last not more than 75 min.
16. The presence of fur may decrease the amount of detectable signal.
17. High doses of isoflurane may affect cellular metabolism in the living subject. The interaction between substrate (D-luciferin) and reporter protein (Firefly Luciferase) requires oxygen, magnesium, and ATP, all cofactors that are available only in viable and metabolically active cells. Therefore, it is critical to avoid deep sedation.
18. Due to its absorption/emission properties, the Renilla Luciferase signal cannot be reliably detected by in vivo BLI. Thus, the use of ex vivo luminometry is recommended.
19. The imaging procedure should not cause any kind of discomfort to the animal.

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Chapter 19

Methods for Long-Term Storage of Murine Bone Marrow-Derived Mesenchymal Stem Cells

Niketa Sareen, Ejlal Abu-El-Rub, Glen Lester Sequiera, Meenal Moudgil, and Sanjiv Dhingra

Abstract

This chapter is based on a simplified method to validate the current preservation procedure of mesenchymal stem cells (MSCs). Currently, there are various media available for freezing and thus preserving the MSCs, making it hard to decide which agent will be apt for cellular requirements. The study describes the effect of two different compositions of freezing media used in regular cell culture experiments, on the morphology, proliferation, and doubling rate of MSCs. Commonly used agents for the cryopreservation of MSCs include DMSO (Dimethyl Sulfoxide) and FBS (Fetal Bovine Serum) and DMEM (Dulbecco's Modified Eagle Medium). To ascertain that the currently used agents do not lead to major changes in the MSC morphology and proliferation, the cells are frozen using the above-mentioned agents in different groups and then their effects analyzed. Thus, the chapter helps to decide what reagents can suit the MSCs, hence minimizing the laboratory to laboratory variability of their characteristics.

Key words FBS, DMSO, Freezing media, Mesenchymal stem cells, Cryopreservation

1 Introduction

Due to their ability to differentiate into various cell types including adipocytes, chondrocytes, and other types, bone marrow-derived MSCs are considered a probable effective treatment for several degenerative diseases [1–3]. In addition to their wide range ability of differentiation into multiple cell types, they possess other characteristics that help in their role as a promised remedy, such properties include secretion of paracrine factors, immunosuppressive abilities, and secretome formation [3, 4]. MSCs exhibit plastic adherent abilities and are similar to fibroblasts in their morphology(spindle shape) with the ease to culture and expand them under in vitro conditions [5]. The long time cultivation of MSCs is not recommended due to its effect on the differentiation and phenotypic characteristics of MSCs and various other factors majorly involving senescence and contamination issues [6, 7].

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Therefore, cryopreservation of MSCs at different passages, preferably early ones, is a very crucial and important step to save these cells and maintain their beneficial characteristics to make a reservoir from healthy MSCs to be used in the future for in vitro and in vivo studies or for clinical applications [7–9]. There are many freezing methods for MSCs, but the most common protocols that exist include: Slow cooling method and Vitrification (rapid cooling method) using high concentration of a cryoprotectant like Me_2SO, glycerol [7, 10]. The slow rate cooling method is the most commonly used and cheap approach for cryopreservation of MSCs. The principle of this method is to reduce the rate of ice crystal formation and osmotic stress that cause injury and damage for the cells, while the temperature is decreasing [7, 10, 11]. DMSO addition to the freezing medium at a concentration range from 5 to 20 % is used for preservation of MSCs using the slow rate cooling method [7, 11]. The use of either FBS with DMSO or the use of FBS and DMEM low glucose medium with DMSO as a freezing solution for MSCs is not determined for their differences in the viability and the attachment of MSCs after thawing. Some studies indicate that the cryopreservation of MSCs using mainly 90 % FCS with 10 % DMSO is the best solution that can be used for the preservation of MSCs [12]. The current study was done to find if addition of medium to the freezing solution enhances the cell viability and proliferation after thawing.

2 Materials

DMEM low glucose medium (with 1g/L D Glucose).

FBS (Fetal Bovine Serum, Heat inactivated by incubating at 56 °C for 30 min) (*see* **Note 2**).

Penicillin Streptomycin (100X).

Beta Mercapto-ethanol (14.3 M).

DMSO (Dimethyl sulfoxide).

MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).

Tissue Culture vessels (Flasks and Dishes).

Pipettes (Glass Pipettes and micropipettes).

All the solutions and media were prepared under sterile conditions in biosafety cabinet in the laminar flow hood.

DMEM Low Glucose complete medium: To 500 ml DMEM low glucose media, add 75 ml of FBS, 5.55 ml of Penicillin Streptomycin, 1.9 μl of beta mercaptoethanol. Store the complete DMEM media at 4 °C.

Freezing Media 1- Add 5 ml of FBS to a 15 ml eppendorf tube; to the same tube add 300 μl DMSO (6 % DMSO).

Freezing Media 2- Add 2.5 ml of FBS in a centrifuge tube and add 2.5 ml of complete DMEM media to the tube containing FBS. Add 6 % of DMSO to the above tube containing media.

MTT dye- Weigh 5 mg of the MTT powder and dissolve it in 1 ml of 1X PBS. The MTT solution is prepared according to the number of tests to be conducted. Thus, prepared solution of MTT dye is stored at 4 °C till further use. MTT dye is light sensitive so the tube containing MTT dye should be covered using aluminum foil and stored.

3 Methods

All the procedures are performed under sterile conditions in laminar flow hood. The medium should be allowed to warm at room temperature before all the experiments.

3.1 Isolation of the Stromal Cells

1. The femur bones are isolated from the hind limbs of the SD (Sprague Dawley) rat species. After isolation, the bones are kept in DMEM media till use (*see* **Note 1**).
2. Using a 10 ml syringe filled with DMEM media, the media is flushed through the isolated bones. This media is allowed to pass into a 50 ml centrifuge tube through a 70 μm sterile cell strainer placed over the tube.
3. The media containing stromal cells is added to a 100 mm petri dish and labeled as P0 SD Rat MSCs along with date of isolation. The dish is then stored at 37 °C in an incubator containing 5 % CO_2.
4. The next day the medium from the petri dish is removed and nearly 10 ml of fresh DMEM low glucose complete medium is added to the dish followed by incubation at 37 °C in CO_2 incubator.

3.2 Passaging of the MSCs

1. Once the dish is nearly 90 % confluent; the medium is removed off the plate and the plate is washed twice with 5–7 ml of calcium and magnesium-free 1XPBS, to remove the residual medium (*see* **Note 3**).
2. To the culture dish containing the cells, 1 ml of trypsin is added and incubated for 5–7 min at 37 °C (*see* **Note 4**).
3. DMEM medium twice the amount of trypsin is added to the dish to inactivate trypsin activity.
4. The plate is gently tapped to remove the adhering cells, and the media with cells is collected in a centrifuge tube.
5. The petri dish is washed twice with the medium to collect the remaining cells.
6. The tube is then allowed to spin in a centrifuge at 23 °C for 5 min at 1500 rpm ($375 \times g$).

7. After centrifugation, the supernatant is removed and the pellet of cells is obtained. The pellet is resuspended in 1 ml of DMEM medium by gentle mixing using a micropipette.
8. To the tube containing resuspended pellet, 9 ml of DMEM media is added. This media with the cells is added to a 100 mm culture dish and incubated at 37 °C in the CO_2 icubator.
9. After the culture dish attains 90 % confluency, passage the cells to fresh culture vessel which has a higher surface area available for attachment of MSCs.
10. The cells are passaged to a desired passage number and once the desired passage stage is attained the cells can be frozen at −80 °C till further use.

3.3 Freezing of MSCs

1. MSCs are trypsinized according to the above-mentioned steps and centrifuged at 1500 rpm (375 × *g*) for 5 min at 23 °C.
2. The cells are counted using an automated cell counter and MSCs are suspended in two different types of freezing media with nearly 1×10^6 cells frozen in 1 ml of freezing solution.
3. The freezing media is added to the vials prelabeled according to the date, passage number, and media used for freezing (*see* **Note 5**).
4. After the addition of the MSCs to the freezing media, the cells are stored at −80 °C in a styrofoambox to attain gradual decrease in temperature for the cells being frozen.
5. The next day the vials with frozen MSCs are transferred from the styrofoambox to the freezer till further use.
6. After seven to ten days of freezing, the vials containing the frozen MSCs are thawed by placing the cryovials in the water bath maintained at 37 °C. Do not allow ice crystal containing MSCs to thaw completely (*see* **Note 6**).
7. Resuspend the frozen MSCs in 1 ml of DMEM complete media. To a centrifuge tube containing 20 ml of DMEM media, add the contents of cryovials. The centrifuge tubes are then allowed to spin at 1500 rpm for 5 min at room temperature (*see* **Note 7**).
8. After centrifugation, the supernatant is discarded and the pellet resuspended in 1 ml of media, followed by spinning again in nearly 20 ml of media under above-mentioned conditions to remove any residual DMSO from the cells. The supernatant is discarded and the pellet resuspended in DMEM media.

3.4 Trypan Blue Cell Viability Assay

1. After uniform mixing of the thawed cells in the media, 10 μl of the media with cells is collected and added to a vial containing 10 μl of trypan blue dye.
2. The cells are mixed uniformly using a micropipette and then 10 μl of the sample is added to the cell counting chamber and the number of viable cells counted. The viability of cells frozen

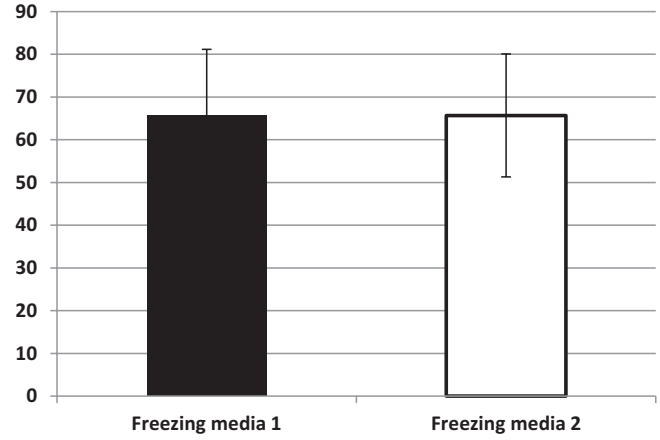

Fig. 1 Trypan blue assay is performed to find the number of viable cells thawed after freezing in two different media. The assay for cell viability indicated same viability for the cells being preserved in either of the media indicating MSCs frozen in media 1 and 2 show equivalent post thaw cell viability

using both the freezing media was similar and thus both the media can be considered suitable in maintaining equal viability after thawing of the cells (Fig. 1).

3.5 MTT Assay for Cell Proliferation

1. Based on the viable cell number, approximately 4×10^4 cells per well are added to a 96-well dish for MTT proliferation assay and 200 µl media is added. The dish is then incubated at 37 °C for 6–24 h.
2. Next day, the media from 96-well dish is discarded and 130 µl of complete DMEM media is added. Add 13 µl of MTT dye to each well containing the cells. Triplicates are maintained for each group. For the blank wells only media and MTT are added to the wells without MSCs. (MTT dye is light sensitive) (*see* **Note 8**).
3. Incubate the dish for 4 h in the CO_2 incubator at 37 °C.
4. After 4 h, the dish is centrifuged at 2000 rpm (666×*g*) for 10 min at 4 °C.
5. After centrifugation, gently remove the supernatant; without disturbing the pellet. To the pellet add 50 µl of DMSO.
6. The plate is then read at 570 nm using a spectrophotometer.
7. The proliferation assay indicates that the viability of MSCs was slightly higher in freezing medium 1 compared to medium 2 (Fig. 2).

3.6 Morphology Analysis

1. In order to analyze the effect of different media on the morphology and phenotype of MSCs, the MSCs are cultured in a 6-well culture dish.
2. MSCs are thawed and counted. The cells are then seeded as 1×10^5 cells per well in a 6-well dish. Three wells are allotted for cells from each freezing media.

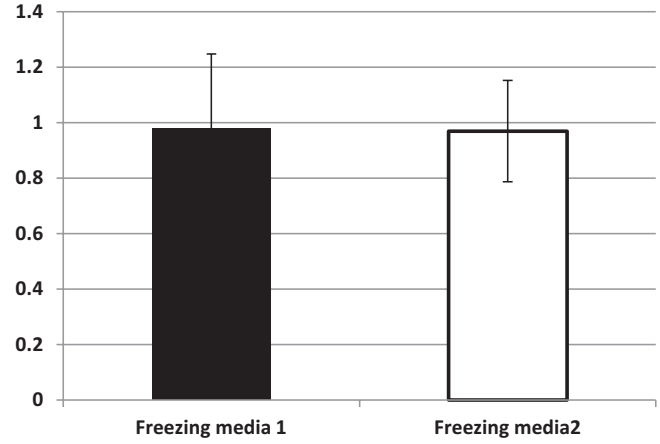

Fig. 2 MTT assay is performed to assess the post thaw proliferation of the MSCs after being frozen in both the media. MTT Proliferation assay indicated faintly increased proliferation in medial 1compared to media 2

3. The next day the media from the wells is removed and fresh DMEM (complete media) media is added.
4. The cells in each group are then imaged using a microscope at different magnifications including 10×, 20×, and 40× (Fig. 3:10× magnification images for analyzing cellular morphology).

4 Notes

1. Once a fresh FBS bottle is being used, it should be inactivated by incubating at 56 °C for 30 min before first use and stored at 4 °C.
2. Media should be warmed to room temperature before using for cell culture.
3. The culture vessels should be washed twice with 1XPBS before trypsinization. Any residual media can lead to trypsin inactivation, thus causing incomplete detachment.
4. Trypsin should be allowed to warm at room temperature before use and for maximal activity the culture vessels should be incubated at 37 °C during trypsin treatment.
5. Once the MSCs are suspended in DMSO containing freezing media, the cells should be frozen as soon as possible to avoid cell deathf as DMSO is harmful for cells.
6. After taking the MSCs from −80 °C, the ice crystal for the frozen cells should not be allowed to melt completely to avoid cell death due to shock.

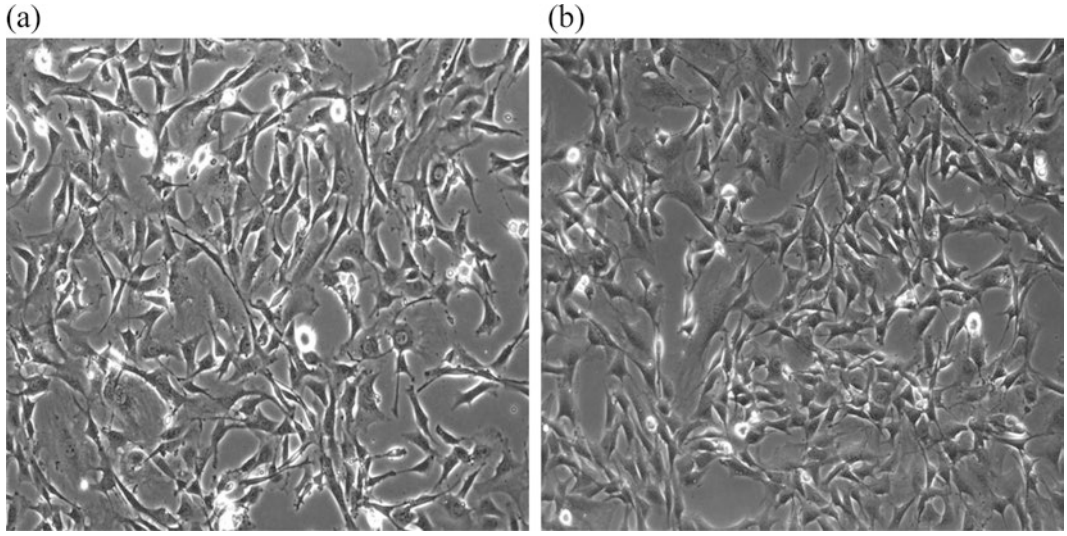

Fig. 3 Post thaw morphological analysis of the MSCs frozen using two different freezing media. (**a**) MSCs frozen using freezing media 1. (**b**) MSCs frozen using freezing media 2 (10X magnification)

7. The DMSO should be removed by centrifugation of the cells after thawing for better attachment.
8. MTT dye should be stored at 4 °C till use and should be protected from light.

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Chapter 20

Therapeutic Application of Adult Stem Cells in the Heart

Taylor A. Johnson and Dinender K. Singla

Abstract

Cell therapies have been explored as a potential treatment avenue to treat heart diseases, such as myocardial infarction, doxorubicin-induced cardiomyopathy, and heart failure. Embryonic and adult stem cells (ASCs) have been examined in animal and clinical settings. Unlike embryonic and induced pluripotent stem cells, ASCs do not pose a threat to form teratomas, nor do they have immune system concerns, making them ideal for therapeutic use in humans. In this review, we will investigate different characteristics and sources of adult stem cells and progenitor cells, as well as determine their efficacy in cell transplantation in experimental and clinical trials. In addition, we will propose other research avenues that may promote further understanding and use of ASCs in therapeutic designs.

Key words Adult stem cells, Progenitor cells, Cardiovascular diseases

1 Introduction

Recent publication on heart disease epidemiological data shows over 85 million Americans have one form of cardiovascular disease, and by the year 2030, projections estimate that nearly 44 % of the US population will develop some form of cardiovascular disease [1]. Since the year 1918, cardiovascular disease accounts for more deaths than any known cause of death in the United States [1]. Aging and heart diseases produce structural changes in the heart that includes cardiomyocyte (heart muscle cell) damage or death, chamber size expansion (known commonly as hypertrophy), tissue scar development, weakening of electrical signals, and impaired blood flow [2–4]. Collectively, these events will impair the function of the cardiovascular system and ultimately hinder quality of life.

Decades of scientific research have uncovered numerous facts and qualities about the heart previously unfathomable. For example, the adult heart has been shown to contain populations of cardiomyocytes that exhibit the ability to proliferate [5]. This is encouraging news, considering that the heart was originally considered a terminally differentiated organ. However, additional

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research on the heart anatomy has also further underscored the need for advanced therapies. It has been shown that less than half of the cardiomyocytes turnover during a normal life span [6] and that cardiomyocyte renewal drastically decreases over time [6, 7].

Due to the rising number of cases associated with various forms of cardiovascular diseases (i.e., stroke, cardiac arrest, atherosclerosis, heart failure), within the United States and worldwide, there are limited heart donors available for transplantation. Therefore, alternative strategies must be explored to generate safe, successful, and financially feasible treatments. In the last decade of scientific advancements, stem cells have been considered to be a safe alternative to treat these heart diseases.

2 Cell Therapy

Cell therapy is the administration of live cells into a patient to treat the disease of interest. Recent therapies have incorporated the use of stem cells; biological cells that have the remarkable ability to self-renew (become the same cell indefinitely in vitro) and differentiate into many cell types. These cells have evolved regenerative medicine into a viable and exciting therapeutic strategy that may delay, stop, or possibly reverse a disease. Patients with an impaired cardiovascular system could be treated with a cell therapy that replaces dead cells, stimulates resident stem cells or angiogenesis, or reduces inflammation and/or fibrosis in damaged tissue. As a result, many animal, preclinical, and clinical trials have been pursued to better understand the effects of these therapies in reversing or reducing damage to the cardiovascular system, as well as promoting cell proliferation, resulting in improved heart/cardiovascular function.

In the next section, we will discuss the three main classes of stem cells: embryonic stem cells, induced-pluripotent stem cells, and adult stem cells. Each of these will be further explored to better understand their characteristics, similarities, and differences, as well as their therapeutic potential.

2.1 Embryonic

Embryonic stem cells (ESCs) are derived from the inner cell mass of embryos at the blastocyst stage of development. These cells have the ability to self-renew and are pluripotent in nature. As pluripotent cells, they can differentiate into any of the three germ layer derivatives (the endoderm, mesoderm, and ectoderm) that give rise to, theoretically, most body cell types [8]. Although ESCs are considered an attractive therapeutic option, due to ethical challenges and limited supply of embryos, this cell therapy approach faces certain challenges. In particular, there is a possibility that upon delivery, ESCs can develop into complex teratomas, a tumor comprised of many tissue derivatives; considered a major limitation in therapeutic use [8–10].

Our lab has studied ESC administration in treating multiple cardiovascular disease models [11–13]. One study evaluated whether transplanted ESCs could repair or regenerated doxorubicin (DOX)-damaged heart tissue. C57BL/6 mice were injected with 1.5×10^6 ESCs and evaluated two weeks post-final injection. Upon administration, there was a notable decrease in apoptotic cardiac nuclei, myofibril loss and cytoplasmic vacuolization, and improved cardiac function [11]. Additional studies have shown that ESCs that overexpress pro-survival proteins TIMP-1 or Thymosin-β4 reduce the number of apoptotic nuclei and fibrosis in mice two weeks post-myocardial infarction (MI) [12, 13].

2.2 Induced Pluripotent

Induced pluripotent stem cells (iPSCs) are generated by harvesting adult stem cells (discussed below) and reprogramming them using different transcription factors. As such, iPSCs can be implemented similarly to ESCs in therapeutics, without having the ethical challenges associated with them. In fact, human iPSCs demonstrated similar differentiation potential, cell morphology, telomerase activity, and expression of cell surface markers commonly found in ESCs [14, 15]. Unfortunately, like ESCs, iPSCs are prone to be tumorigenic in nature [16].

Our lab has successfully generated iPSCs and evaluated their efficacy in multiple cardiovascular disease models [17–19]. Using four essential factors (Oct3/4, Klf4, c-Myc, and Sox2), cardiomyoblast H9c2 cells were reprogrammed into beating cardiomyocytes in vitro. Generated iPSCs, ESCs, or H9c2 cells were then administered into C57BL/6 mice after MI (5×10^4 cells) and evaluated two weeks thereafter. iPSC transplantation was shown to be comparable to ESC treatments in reducing apoptosis and fibrosis, ultimately improving cardiac function [17]. A separate study evaluated the therapeutic use of iPSCs in streptozotocin (STZ)-induced diabetic cardiomyopathy. Three injections of iPSCs (4×10^5) were administered post-final streptozotocin injection in mice. Two weeks post-STZ, iPSC administration was shown to reduce apoptosis, fibrosis, and pro-oxidant expression and promote antioxidant activity [18]. The effects of iPSCs were examined in treating DOX-induced heart failure following myocardial infarction. C57BL/6 mice were subjected to cumulative doses of DOX and subsequently MI two weeks later. Post-MI, 5×10^4 iPSCs or ESCs were administered and mice were sacrificed 2 weeks later. Both stem cell therapies were shown to reduce vascular and interstitial fibrosis and improve heart function [19]. Collectively, these studies show that transplanted iPSCs reduce cardiac remodeling and improve heart function.

2.3 Adult

Adult stem cells (ASCs), also known as somatic stem cells, are undifferentiated, self-renewing multipotent cells. ASCs function by differentiating into cells that will maintain and repair the tissue

those cells reside in (i.e., ASCs in the heart become cardiac stem cells (CSCs)). Since ASCs are derived from the host, there is no immune response; however, adult stem cells have limited multipotency, compared with ESCs or iPSCs. Recent research, however, has demonstrated the ability for ASCs to transdifferentiate. Transdifferentiation allows for one somatic cell type to be directly manipulated into becoming another somatic cell type. There are two different methods that induce transdifferentiation: natural and experimental. Natural transdifferentiation requires the desired cell to naturally dedifferentiate into a precursor stage and concurrently differentiate into another cell type simultaneously. This is achieved by the inactivation of the cells original factors and the activation of factors that promoted the desired cell fate. For example, in newts, pigmented epithelial cells have been documented to transdifferentiate into new, mature lens cells to replace missing lens eye tissue [20]. This is possible through the inactivation of retinoblastoma protein and the expression of developmental genes (i.e., pax6; prox1) [20]. In contrast, experimental transdifferentiation requires that the scientist stimulate transdifferentiation by manually manipulating the activity of different factors (i.e., using transcription factors to transdifferentiate fibroblasts into immature neurons or cardiomyocytes) [20].

3 Source of Adult Stem Cells

Adult stem cells are found in most of the body's organs; however, they have been shown to reside in localized areas called stem cell niches. Numerous stem cell niches have been identified, such as neural stem cells within the anterolateral ventricle and hippocampus of the brain [21], mesenchymal and hematopoietic stem cells from bone marrow [22], epidermal stem cells in the skin [23], adipose stem cells from fat tissue [24], and stem cells from skeletal muscle [25] and the liver [26].

Figure 1 displays several locations of adult stem cell niches found with the body commonly used in research, including the bone marrow, brain, and heart. Recently, research has shown additional adult stem cells located in hair follicles [27], reproductive organs [27], teeth [28], alimentary canal [29], and within circumventricular organs and the third and fourth ventricles of the brain [21].

4 Adult Stem Cells and Progenitor Stem Cells

Progenitor cells, also known as precursor cells, are lineage specific cells that have a tendency to differentiate into a specific cell type, compared with adult stem cells, which can self-renew and

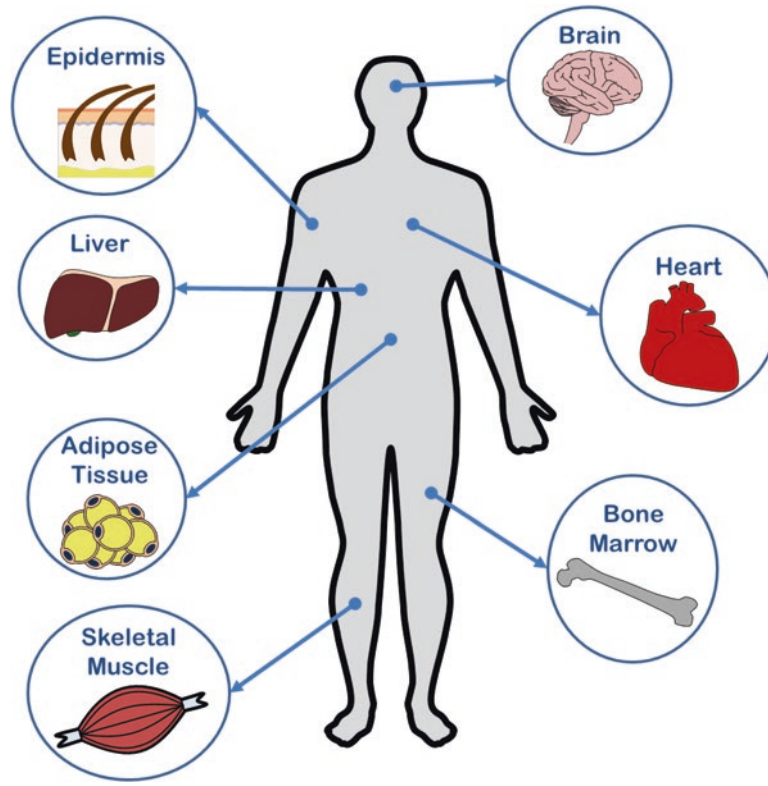

Fig. 1 Locations in the body where adult stem cells are commonly harvested for study

differentiate into multiple cell types. The classic examples distinguishing stem cells and progenitor cells are bone marrow stem cells and common lymphoid/myeloid progenitor cells. Bone marrow stem cells can self-renew or differentiate, whereas lymphoid and myeloid progenitor cells give rise to further differentiated cells. Lymphoid progenitors become either T cells, natural killer (NK) cells, B cells, or dendritic cells [30, 31]. Additionally, myeloid progenitor cells can be further differentiated into two other progenitors; one progenitor gives rise to erythrocytes (red blood cells) and megakaryocytes (platelet production) and the other granulocytes, monocytes or dendritic cells [30, 31].

Endothelial progenitor cells have recently been evaluated as a therapeutic method to help the cardiovascular system regain function after injury. Xu et al.'s review article details early- and late-endothelial endothelial progenitor cells that secrete a myriad of factors, including VEGF, IL-8, FGF, HGF, SDF-1, Thymosin β4, and IGF. Moreover, these secreted factors contributed to endothelial cell proliferation, improved angiogenesis, and cardiac function [32].

Multiple cardiac progenitor cells have been identified in the cardiovascular system. These progenitors include C-kit-positive

($^{+ve}$), Sca-1^{+ve}, Isl-1^{+ve}, side population (SP), cardiospheres, and epicardium-derived progenitor cells. These progenitor cells have been extensively reviewed here [33–36], and have been shown to improve cardiac function following transplantation.

5 Stem Cell Transplantation in Experimental Conditions

Stem cell therapies have been studied in numerous animal models, including drosophila [37], the roundworm *Caenorhabditis elegans* [38], zebrafish [39, 40], mice [41], and rats [42–44].

Zebrafish are commonly used for heart regeneration studies, due to strong regenerative response upon injury. After 20 % ventricular resection, the zebrafish heart fully replenished [39, 40]. Without stem cell transplantation, mature zebrafish cardiomyocytes will undergo dedifferentiation and transdifferentiation [40, 45]. At this time, the mechanisms of zebrafish heart repair and endogenous stem/progenitor cell regeneration potential remain unknown.

Multiple ASC types were compared to one another upon administration into MI-induced mice. *Female* FVB mice were injected in the heart with either bone marrow mononuclear cells, mesenchymal stem cells, skeletal myoblasts, or fibroblasts (5×10^5) cultured from *male* L2G mice bred on fluorescent protein background. Using bioluminescence imaging, cells were able to be tracked once injected into the mice. Bone marrow mononuclear cells were shown to migrate into other organs, such as the spleen and femur, over a 4 week period. In contrast, signals of other cell types strongly diminished within 2 weeks. PCR of the Sry locus (found only on the Y chromosome) confirmed a dramatic increase of bone marrow mononuclear cells within the heart 6 weeks post-injection. In addition, mice administered with bone marrow mononuclear cells exhibited the highest % fractional shortening 4 weeks and 6 weeks post-injection, compared with other treatments. Collectively, this comparative study showed between multiple ASC treatments, bone marrow mononuclear cells best improved overall cardiac function in mice [41].

Skeletal muscle derived stem cells (Sk-34 cells) were investigated in vitro and in vivo in a rat MI model. When cocultured with embryonic cardiomyoblasts, high expression of cardiomyogenic transcription factors was observed, in addition to gap-junction and synchronized contraction following colony formation. In vivo, nude mice were injected with ($5–8 \times 10^5$) Sk-34 cells post-MI. 4 weeks post-injection, sk-34 administration contributed to intercalated disc formation, resulting in improved cardiac function [42].

Cardiac progenitor cells (1×10^6) were administered to female Fischer 344 rats 30 days post-MI. 35 days post-treatment, cardiac

progenitor cell treated groups showed increased myocardium within the scar area, less fibrosis, and improved cardiac function. Interestingly, only 7 of the 17 treated rats observed had cardiac progenitor cells located within the heart. Upon injection of exogenous cardiac progenitor cells, endogenous cardiac progenitor cells within the rat were activated and traveled to the site of injury [43].

6 Preclinical Swine Studies

Multiple studies evaluated bone marrow-derived mesenchymal stem cells (MSCs) as a potential therapy using MI-induced swine models [46–48]. MSCs post-administration were shown to engraft into infarcted areas [46, 48]. In addition, MSCs differentiated into cardiomyocytes, smooth muscle cells, and endothelial cells [46, 48]. Upon administration, cardiac function improved, as demonstrated by decreased infarct size and improved % ejection fraction (EF) [46, 47]. Cardiac MRI studies revealed that MSC administration prevented progressive dilation of end-diastolic volume and end-systolic volume of hearts 6 months post-injection [47]. In addition, MSC administration stimulated a 20-fold increase of endogenous C-kit^{+ve} CSCs compared with control animals [48].

Hibernating myocardium, due to repetitive ischemia and impairment of contractility, was stimulated in swine using an occluder in the proximal LAD. Thereafter, swine received injections of bone marrow-derived MSCs (4.4×10^7 cells). Post-treatment, MSCs stimulated increases in bone marrow progenitor cells and cardiomyocytes in hibernating hearts, resulting in improved cardiac function. Although the mass of the left ventricle increased, it was noted that the myocyte size was smaller, which supports the idea of endogenous cardiomyocyte proliferation upon treatment [49].

Autologous bone marrow stromal cells were compared to autologous heart cells in restoring function after MI. Four weeks post-infarction, using as single-photon emission tomography (s-PET), both treatments improved heart perfusion, whereas non-cell-treated hearts decreased. In addition, both treatments improved cardiac function, noted by increases in end systolic pressure and preload-recruitable stroke work. Histologically, both treatments groups demonstrated reduced scar areas and stromal cells were shown to differentiate into endothelial cells that contributed to blood vessel formation [50].

C-kit^{+ve} cardiac stem cells were injected into MI-induced swine in two separate studies [51, 52]. C-kit^{+ve} CSCs treated animals were shown to have higher ejection fraction and reduced end-diastolic pressure [51, 52]. CSCs administration (5×10^5) was shown to stimulate cardiomyocyte proliferation and the formation of vascular structure [51]. When C-kit^{+ve} CSCs were combined

with MSCs (1×10^6 and 2×10^8 respectively), MI size reduction was twofold greater than that of C-kit^{+ve} only or MSC only treatments [52]. In addition, combined CSC/MSC treatment demonstrated higher % EF, lower end-diastolic pressure, and a sevenfold engraftment of stem cells in the myocardium compared to single cell treatment [52]. This data suggests that combination therapy of CSCs and MSCs is superior compared with individual cell type therapy.

7 Human Clinical Trials

Table 1 shows several human trials utilizing cell therapies for improvement of heart function. TAC-HFT, the Transendocardial Autologous Cells in Ischemic Heart Failure Trial, was a Phase I/II trial evaluating the effects of bone marrow-MSCs (BM-MSCs) and mononuclear bone marrow cells (mBMCs) in patients with ischemic cardiomyopathy and % EF less than 50 % (NCT00768066). Patients were injected in 10 left ventricle sites using an infusion catheter with either a cell treatment or a placebo and evaluated 1 year later. Infarct size was shown to be reduced upon MSC administration but not by mBMCs or placebos. In addition, 6-min walk distance (6MWT) increased only in the MSC group. Patients who received cell-based treatments had reduced MLHF and New York Heart Association (NYHA) scores. In addition, patients who received with BM-MSCs or mBMCs had a reduced incidence of serious adverse events (~32 %) compared to placebo (~38 %). Serious adverse events are defined nonfatal MI, pericardial tamponade, ventricular arrhythmias, stroke, worsening heart failure, and composite of death [53]. TAC-HFT II, a clinical trial evaluating two different autologous treatments (1. human MSCs and 2. human MSCs cotreatment with human C-kit^{+ve} CSCs), will begin within the next few years (NCT02503280).

POSEIDON, the Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis Pilot Study trial, was a phase I/II trial examining the effects of BM-MSCs harvested from autologous (same patient) and allogeneic (healthy donor) in repairing MI hearts (NCT01087996). Similar to that of TAC-HFT, cells were delivered using a catheter into 10 left ventricle sites. After 1 year, the incidences of serious adverse events were much lower in allogeneic-treated patients (33.3 %) compared to autologous patients (53.3 %). Low-dose concentrations (20 million cells) were shown to provide the greatest improvement in ejection fraction, compared to higher dosages (100 or 200 million cells) [54]. A follow-up trial (POSEIDON in Dilated Cardiomyopathy; POSEIDON-DCM) is currently ongoing (NCT01392625) [55].

The PROMETHEUS trial, Prospective Randomize Study of Mesenchymal Stem Cell Therapy in Patients Undergoing Cardiac Surgery, examined six patients who had ischemic cardiomyopathy

Table 1

Human clinical trials of patients with prior history of ischemic cardiomyopathy or myocardial infarction. Abbreviations: 6MWT—6 Minute Walk Test;al.—Allogeneic;at.—Autologous;BM-MSC—Bone Marrow-Mesenchymal Stem Cell; CABG—Coronary Artery Bypass Grafting;CPC—Circulating Progenitor Cells;CVD—Cardiovascular Disease;EF—Ejection Fraction;IMP—Improvement;IS—Infarct Size;mBMC—mononuclear Bone Marrow Cells;MLHF—Minnesota Living with Heart Failure Score;NYHA—New York Heart Association Functional Class;RDT—Reduction;ICMP—Ischemic Cardiomyopathy;STEMI—Acute ST-elevation Myocardial Infarction;SK-MB—Skeletal Muscle Myoblast

Trial name	CVD	Cell type	# Patients	% Males	Age Mean	6MWT IMP?	NYHA IMP?	MLHF IMP?	IS RDT?	EF IMP?	Source
TAC-HFT	ICMP EF < 50 %	at. BM-MSC at. mBMC	59	~ 93 %	~ 60	Yes No	Yes Yes	Yes Yes	Yes No	No No	[53]
POSEIDON	ICMP EF < 50 %	at. BM-MSC al. BM-MSC	30	~ 87 %	~ 63	Yes Yes	Yes Yes	Yes Yes	Yes Yes	Yes Yes	[54]
PROMETHEUS	ICMP Undergoing CABG	at. BM-MSC	6	100 %	~ 55	N/A	N/A	N/A	Yes	Yes	[56]
TOPCARE-AMI	STEMI	at. mBMC at. CPC	55	N/A	~ 52	N/A	No	N/A	Yes Yes	Yes Yes	[57]
FINCELL	STEMI	at. mBMC	80	~ 88 %	~ 60	N/A	N/A	N/A	N/A	Yes	[58]
MAGIC	EF < 35 %; MI Undergoing CABG	at. SK-MB	97	~ 96 %	~ 60	N/A	No	N/A	N/A	Yes	[59]

and were undergoing coronary artery bypass grafting (CABG) (NCT00587990). 18 months post-treatment with autologous BM-MSCs, patients had increased % EF and a reduction in scar tissue. In addition, there were positive correlations between the percent of scar tissue that reduced over time and (1) the number of cells injected (up to 200 million per segment) and (2) the number of injections per segment (up to 10 injections) [56].

The TOPCARE-AMI trial, Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction, compared two cell therapies from different sources; autologous mBMCs from bone marrow and circulating progenitor cells (CPCs) from the blood. 5 years post-intracoronary injection of cells, ejection fraction was significantly improved in patients with acute ST-segment elevation MI (STEMI) treated with coronary stenting. In addition, left ventricular mass and infarct size were reduced in both groups. However, neither group improved their NYHA score nor was there a dramatic difference when comparing the number of adverse cardiac events [57]. A second study, the FINCELL (Finnish stem cell) study, also evaluated patients with previous STEMI conditions. 6 months post-intracoronary injection of mBMCs, patients demonstrated increased ejection fraction, due to a reduction in end-systolic volume in the left ventricle [58].

MAGIC, the Myoblast Autologous Grafting in Ischemic Cardiomyopathy trial, enrolled patients with myocardial infarction, left ventricular dysfunction (less than 35 %) and underwent CABG. Patients underwent skeletal muscle biopsy for the culture of skeletal muscle myoblasts (SK-MBs) and were injected back into the patient's heart. 6 months post-injection, the absolute change in % EF between the placebo, low-dose treatment (400 million myoblasts), and high-dose treatment (800 million myoblasts) were similar. However, there were significant decreases in left ventricle volume during both diastole and systole in the high-dose group [59].

In addition to using science-based techniques to evaluate efficacy of a treatment, numerous survey-based measurements were implemented. For example, the Minnesota Living Heart Failure (MLHF) score is a response questionnaire used to evaluate the effects of heart failure on a patient's quality of life. It measures the effects of symptoms, psychological distress, and function limitations due to treatment [60].

8 Reflection on Human Studies

Collectively, majority of the studies conducted focus on the use of bone marrow stem cells as a therapeutic option. Many of the bone marrow based therapies were shown to improve heart function. Neither BM-MSC or mBMC treatments improved % EF in the TAC-HFT trial; however, both treatments were effective in all

other trials. In addition, only the mBMC-treated patients in the TAC-HFT trial did not see reduction in infarct size. Therefore, the use of adult stem cell transplantation in clinical trials remains inconclusive.

Researchers and medical professionals should be cautious when utilizing bone marrow cells for potential heart/cardiovascular disease therapies. Based on recent studies using mice and rat models, unselected bone marrow cells or bone marrow-extracted mesenchymal stem cells could form calcification within the heart following transplantation [61, 62].

9 Factors that Activate Endogenous Adult Stem Cells

It has been reported that administration of stem cells or progenitor cells can stimulate activation of endogenous adult stem cells in the host. Immunodeficient mice were subjected to myocardial infarction and administered human endothelial progenitor cells (5×10^5) [63]. Two weeks post-injection, the expression of human EPC-derived cytokines decreased and was replaced by upregulation of host cytokines that mobilize bone marrow cells. Human EPC transplantation resulted in movement of endogenous stem cells or progenitor cells from the host's bone marrow to the MI site [63].

In vitro, prolonged treatment of mesenchymal stem cells with HGF stimulates the expression of multiple cardiac markers (i.e., GATA-4; α- and β-MHC) [64]. In addition, Urbanek et al. have shown that C-kit^{+vc} CSCs can synthesize and secrete c-Met and IGF-1 as well as their respective ligands (HGF and IGF-1 respectively). Mice were subjected to myocardial infarction and treated with 4 injections of combined HGF/IGF-1 therapy. Treatments resulted in recruitment of other C-kit^{+vc} CSCs, stimulating the growth of arterioles, capillaries, and myocytes within the new myocardium tissue. New myocytes were shown to effectively mature and grow in size and cardiac function greatly improved with treatment [65].

As reviewed by Gopinath and Rando, satellite cells may be influenced by surrounding stem cells in the vasculature as the skeletal muscle ages [25]. The secretion of factors from the endothelium, such as VEGF, IGF-1, and eNOS, could influence satellite cell expansion or efficacy [25]. Further studies are required to evaluate other stem cell populations and their potential ability to influence other stem cells.

10 Future Direction

Intensified research of adult stem cell-based therapies is vital for the development of novel therapeutics aimed to treat patients with heart and cardiovascular diseases. A holistic approach needs to be

taken to better understand and treat diseases; this includes better comprehension of molecular and cellular mechanisms during normal and diseased conditions, innovative approaches for designing potential cell-based therapies and effective translation and execution of those therapies in humans.

Direct cell delivery into the damaged area can result in significant cell loss, as cells could be flushed out of the area by the circulatory system. In addition, the cell death rate can be influenced by where and how strongly cells are grafted to tissue [66]. As such, additional avenues must be evaluated to determine the best mode of administrating cell therapies. The combined use of biomaterials and cell therapies has become increasingly more attractive, especially with advances in material science and biomedical engineering.

Recently, we reviewed the potential of stem cells in three-dimensional bioprinting, which should be biologically safe [67]. A future alternative in regenerative medicine, this will allow for the improvement and/or replacement of damaged tissues.

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Chapter 21

Patient-Derived and Intraoperatively Formed Biomaterial for Tissue Engineering

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Abstract

In this chapter, we introduce a completely intraoperative procedure for obtaining a patient-derived biomaterial in cell therapy and tissue engineering applications. An automated device for processing human peripheral-blood ensures a reproducible method for retrieving the patient's cellular-rich as well as cellular-poor plasma. By substituting calcium for animal-derived thrombin, we engineer a completely autologous hydrogel that eliminates the risk of disease transmission and lowers FDA regulation hurdles. Through this chapter, we will discuss a bedside protocol developed to prepare a patient-derived hydrogel. This method can be effectively used to develop a completely intraoperative tissue engineering strategy (CITES) that can be easily translated into the clinic for surgical use.

Key words Intraoperative, Peripheral blood, Automated device, Cell therapy, Tissue Engineering, CITES

1 Introduction

Tissue engineering strategies involve biomaterials, cells, and signaling molecules, either alone or in combination [1, 2]. The development and discovery of biomaterials as matrices for cell loading and growth have led to great advancements in the field of tissue engineering [3–6]. Hydrogels are developed for this particular application, as they offer a three-dimensional environment for cell growth, delivery and retention, water for hydration, and tissue-like mechanical behavior [7–9]. Primarily, hydrogels have been classified as either synthetic or natural, based on the biomaterial used to form the hydrogel [1, 10]. Polyethylene glycol, polyvinyl alcohol, and poly alpha-esters are some of the commonly used synthetic hydrogels [11–13]. They offer synthetic flexibility and property modulation,

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which is the key to designing hydrogel matrices with specific physical and mechanical properties [1, 8].

Natural hydrogels are widely used in tissue engineering due to their excellent biocompatibility [14–16]. Developed hydrogels have been part of numerous drug delivery and tissue engineering strategies. Some known examples of natural hydrogels include collagen, elastin, and fibrin, among which fibrin stands out as it can be derived from the patient itself [17–22]. Fibrin has been known and used as a sealant for more than 50 years [23]. Fibrinogen protein is combined with animal-derived thrombin to form fibrin hydrogel [24–26]. Although the animal-sourced thrombin (bovine) effectively converts fibrinogen to fibrin, it carries the risk of disease transmission [27] and has been restricted in many European and South America countries. The fibrin-containing tissue-engineered device also requires FDA approval before surgical use [27].

Therefore, there is renewed interest in patient-derived biomaterials, which, by virtue, are derived from the patient and can be implanted into the same patient. Patient-derived biomaterials can be developed within clinical practice, and thus, unlike existing materials, are not subject to the FDA approval process [28]. To our knowledge, there are only two such materials: blood-derived fibrinogen, and hair-derived keratin [19, 29]. In this article, we have developed a completely automated protocol to isolate and form fibrin hydrogel from the patient's peripheral blood. An automated device is used to obtain plasma from the blood in a reproducible manner [30, 31]. Moreover, this protocol develops a method to gelate plasma into fibrin without the use of thrombin. Overall, this methods chapter will present a completely intraoperative protocol to derive fibrin from peripheral blood next to the bedside for tissue engineering use.

2 Materials

1. Peripheral Blood Sample: Obtain 30–40 mL of donor blood (*see* **Note 1**).
2. Autologous Platelet Separator System: We use the Arteriocyte Magellan® System that is complete with a fully automated self-balancing centrifuge and disposable separation chamber.
3. Two 60 mL syringes (provided with Magellan® processing kit).
4. Anticoagulant to coat syringes: Anticoagulant Citrate Dextrose Solution, Solution A (ACD-A).
5. Calcium chloride to form gel: 100 mM in 5 mL stock solution.
6. Pipettes and five aliquot tubes.

3 Methods

The protocol is implemented as per the schematic presented in Fig.1. All the procedures are performed at room temperature, unless indicated otherwise.

3.1 Obtaining Plasma

1. Autolgous Human peripheral blood can be drawn into one of the anticoagulated 60 mL syringes. For this development, we obtained human peripheral blood from a commercial source (Zenbio Inc.) that was shipped in a tube containing anticoagulant on the same day the blood is drawn, and is received the day after.
2. Take 60 mL syringes and rinse them with ACD-A solution (anticoagulant). Pipette the solution up and down to coat the inside surface of the syringe in ACD-A, and then recycle the remaining ACD-A solution.
3. Transfer the blood sample into one of the ACD-A-coated syringes. Keep the second syringe empty for later use (*see* **Note 2**).
4. The Instructions for Use of the Magellan® System are included with each disposable kit. Briefly, open both the centrifuge and syringe covers to load the device. Place the disposable separation chamber into the caddy of the centrifuge by first maneuvering the tubing through the canal of the base. Lock the separation chamber in place by gently pushing on both ends of the cylindrical surface and funnel the tubing through the tubing clamp and grooved fixture leaving the centrifuge housing. Close centrifuge cover and look for green light to indicate successful locking. Attach the long and the short tubing to the provided 60 and 10 cc BD syringes, respectfully (*see* **Note 3**). Funnel long tubing through the pinch-valve under syringe cover. Place syringes into the syringe handles and close syringe

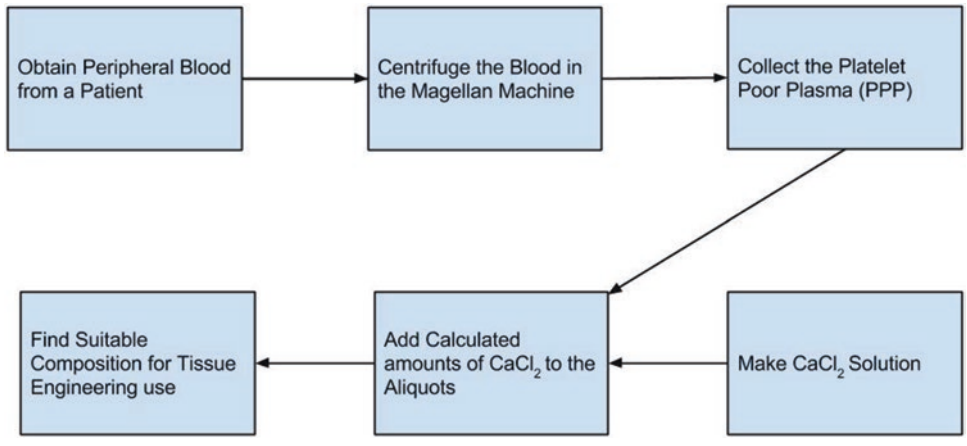

Fig.1 Schematic showing the steps utilized to prepare fibrin gel from human blood-derived plasma. Plasma was obtained by processing peripheral blood via Magellan® System followed by calcium-mediated gelation

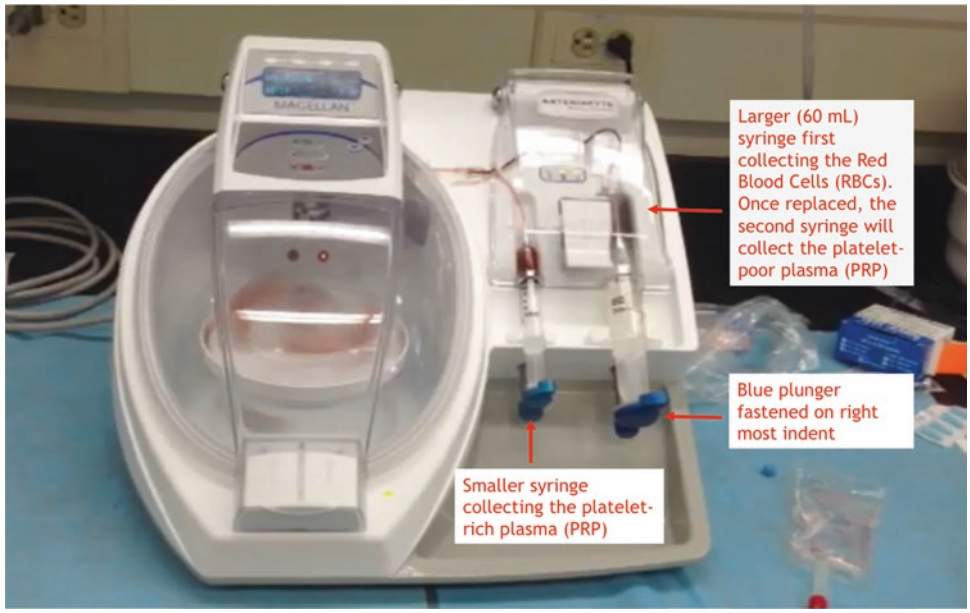

Fig.2 Magellan® System used to process human peripheral blood—it is a completely automated and close-looped device with the ability to separate blood into three fractions: red blood cells, platelet-rich plasma, and platelet-poor plasma. The latter fraction was used to form fibrin gel by mixing with an appropriate amount of calcium chloride solution

cover. Green light will indicate successful assembly and closure (*see* **Note 4**).

5. Select the final desired volume of concentrate (3–10 mL) using the "+" and "−" buttons. Press the green "play" button and then "PPP" to begin processing the blood. The Magellan® System is a dual spin processing device. After a soft spin to concentrate a packed Red Blood Cell (RBC) layer, the packed RBCs will be removed and collected back into the existing 60 mL BD syringe. While the device enters into the hard spin to create the PRP, remove the RBC-filled syringe and replace with the empty 60 mL BD syringe previously coated with ACD-A and press start again. This new syringe will collect the Platelet-poor Plasma (PPP) to be used for the following steps. Dispose of the first syringe appropriately. Once the cycle has been completed, remove the syringe from the machine. PRP will be collected into the 10 mL BD syringe, as shown in Fig.2 (*see* **Note 5**).

3.2 Creating the $CaCl_2$ Mixture

1. Obtain five aliquot tubes. Each of these tubes will contain the same amount of PPP (referred to from now on as plasma), but various concentrations of 100 mM calcium chloride, to determine the optimal concentration of calcium for gel formation. Weigh out the appropriate amount of 100 mM $CaCl_2$ needed for a 5 mL stock solution.
2. Determine five different concentrations of $CaCl_2$ and label each of the aliquot tubes with the corresponding volume.

3.3 Plasma Gelation to Form Fibrin

1. Add 100 μL of the plasma to the first aliquot tube (*see* **Note 6**).
2. Add the appropriate volume of $CaCl_2$, and immediately start the timer. Begin to gently rotate the tube toward a 90° angle. The point at which a distinct difference between gel and liquid is observed is the time required for gelation (*see* **Note 7**).
3. Other than the point at which the liquid is seen to diffuse through the forming gel, another important observation is when the tube is completely flipped over (180°), and the gel sticks to the top (*see* **Note 8**).
4. Repeat this procedure (**steps 1–3**) for the remaining five aliquot tubes, and record the time required for gelation.

3.4 Data Analysis

1. Analyze the time required for gelation of each of the aliquot tubes, and determine the optimal concentration of $CaCl_2$. Depending on who is performing the experiment, the optimal value may be different (*see* **Notes 9** and **10**).

4 Notes

1. The Magellan® System needs about a minimum of 30 mL to operate; however, optimal results are achieved with 40–60 mL of starting volume.
2. The purpose of adding ACD-A to the syringe is to hinder the process of blood clotting. Therefore, using a syringe that has not been coated with ACD-A could result in early gelation and skew your results.
3. The small 10 mL BD syringe needs to be connected to the tubing and placed into the syringe position for the device to perform. Platelet-rich plasma (PRP) is collected in this syringe. ACD-A is not needed in the 10 mL syringe (Fig. 2).
4. Light indicates when the system has been appropriately loaded. Any errors in assembly will be indicated by red light instead of green.
5. The Magellan® System mainly functions based on the principle of density separation [30, 31]. Erythrocytes are the most dense, followed by platelets, leukocytes, and plasma. Platelet-rich plasma is more dense than platelet-poor plasma since it contains more cells. Rapid spinning of the peripheral blood separates it into various layers based on density. Red blood cells and granulocytes, being the most dense, are collected into the syringe first. After replacing the filled syringe, the less dense platelet-poor plasma is collected into the second syringe. The platelet-rich plasma is simultaneously collected in the smaller syringe.
6. Pipette the plasma up and down before transferring it to the aliquot tube to ensure a homogenous and even amount of fibrinogen in each tube.

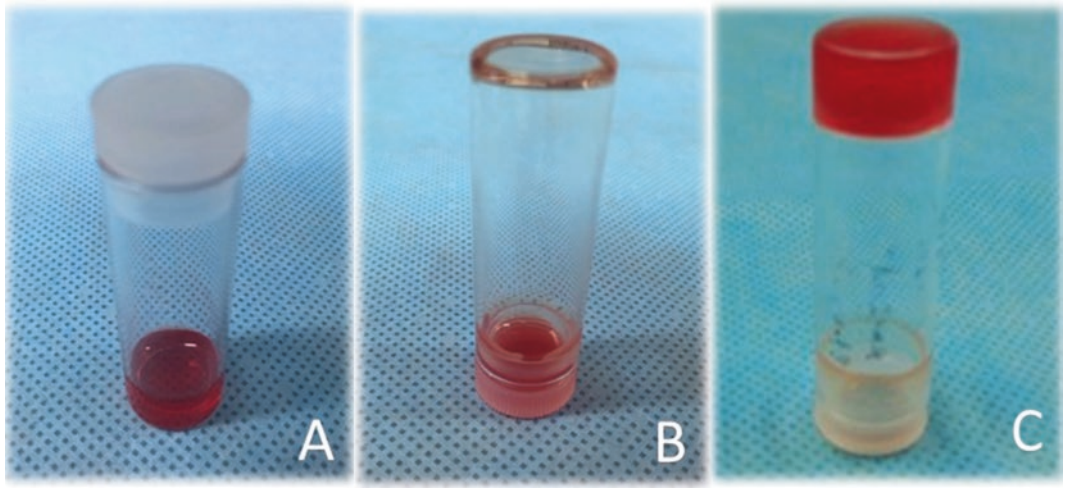

Fig.3 Human peripheral blood derived plasma before and after gelation: (**a**) Plasma in tube, and (**b**) Plasma inverted 180° before gelation. (**c**) Plasma and $CaCl_2$ mixture flipped over 180° after gelation period shows that gelation has occurred. The hydrogel is viscous and adheres to the top surface

Table 1
Determining optimal calcium chloride concentration required for plasma gelation. Increasing calcium chloride concentration results in faster gelation time. Calcium chloride concentration of 15 mM/L gives approximately 120 s to transfer the plasma and calcium mixture out of the tube

Calcium chloride (mM/L)	Approximate gelation time (s)
15	120
25	90
35	80
45	50
55	40
65	30
75	10

7. Do not miss the window of the initial gel formation. This is the critical time that differs between various calcium concentrations.
8. At this point, a significant amount of gelation has occurred such that the substance "sticks" to the top (Fig. 3).
9. In a real-world application, the optimal concentration of $CaCl_2$ is that which gives the surgeon enough time to transfer the mixture of plasma and calcium into the body before gelation has initiated (Table 1).
10. Human Mesenchymal Stem Cells (HMSCs) suspended in the hydrogel were observed for 21 days to ensure that the patient-

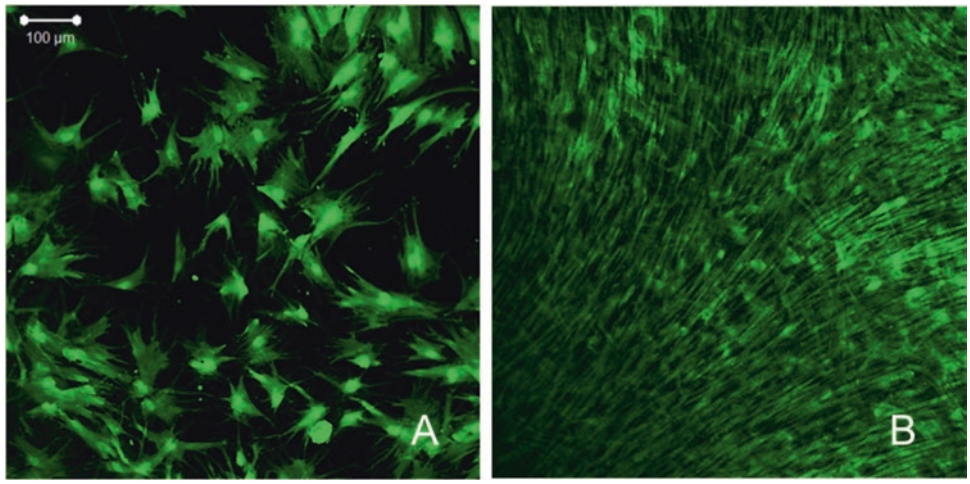

Fig.4 Confocal microscope images of human bone marrow stromal cells (hBMSCs) in fibrin hydrogel cultured for: (**a**) 1 day, and (**b**) 14 days. After 1-day culture, hBMSCs appear to spread with cell processes extended in all directions. By day 14, cells became confluent. These images suggest that a patient-derived and calcium mediated fibrin hydrogel supports cell survival and growth

derived fibrin supports cell survival and growth. A confocal microscope picture of the cells shows that the environment supports cell survival and growth (Fig. 4).

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Chapter 22

Three-Dimensional Printed Scaffolds with Multipotent Mesenchymal Stromal Cells for Rabbit Mandibular Reconstruction and Engineering

Dongdong Fang, Michael Roskies, Mohamed-Nur Abdallah, Mohammed Bakkar, Jack Jordan, Li-Chieh Lin, Faleh Tamimi, and Simon D. Tran

Abstract

Multipotent mesenchymal stromal cells (MSC) derived from both the bone marrow and adipose tissue possess the ability to differentiate into multiple cell lineages, regulate the immune function by secreting numerous bioactive paracrine factors, and hold great potential in cell therapy and tissue engineering. When combined with three-dimensional (3D) scaffolds, MSC can be used for bone defect reconstruction and engineering. This protocol describes the isolation of bone marrow mesenchymal stromal cells (BMMSC) and adipose-tissue derived stem cells (ADSC) from rabbits for subsequent seeding on tissue-engineered 3D-printed scaffolds and transplantation into a rabbit-model with the goal of repairing large osseous mandibular defects (one quarter of the lower jaw is removed surgically). Steps to demonstrate the three cell differentiation lineage potentials of BMMSC and ADSC into osteocytes, adipocytes, and chondrocytes are described. A modified cell seeding method using syringes on scaffold is detailed. Creating a large mandibular bone defect, the rapid prototyping method to print a customized 3D-scaffold, the scaffold implantation procedure in rabbits, and microcomputed tomography (micro-CT) analysis are also described.

Key words Rabbit bone marrow mesenchymal stromal cells, Rabbit adipose-tissue derived stem cells, 3D scaffold, Mandibular reconstruction, Tissue engineering

1 Introduction

The mandible is critical for the facial appearance/harmony and functions, such as mastication, swallowing, and speech [1]. Mandibular defects result from trauma, infections, or after surgical resection of tumors [2]. Autogenous vascularized bone grafts, such as fibular free flaps [3] or iliac flaps [4], are most commonly used to reconstruct the mandible, since they offer several advantages, including consistent shape, sufficient blood supply, ample length, and low donor-site morbidity. However, autogenous bone harvesting is often associated with a number of complications, including

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infection, hematoma, fracture, and nerve injury [4]. Additionally, thin fibula has proven a poor match for mandibular height, leading to a potential loosening of dental implants.

Recent studies suggest that scaffold biomaterials can be used as alternative materials to reconstruct critical-size bone defect [5]. Three-dimensional (3D) printed scaffolds can be customized and precisely printed based on the CT or MRI 3D picture files of patients. However, it was reported that osteogenesis only occurred in the outer surface of large scaffolds, leading to a nonhomogenous distribution of cells [5]. To overcome these limitations, mesenchymal stromal cells (MSCs) were seeded in porous scaffolds and were tested for the reconstruction of bone defects [6, 7].

MSCs derived from both bone marrow [8, 9] and adipose tissue [10–12] have a great potential in cell therapy and tissue engineering, since they possess the ability to differentiate into multiple cell lineages, and regulate immune function by secreting numerous bioactive paracrine factors. Bone marrow mesenchymal stromal cells (BMMSCs) have demonstrated osteogenic differentiation both in vitro and in vivo. So far, bone marrow is considered the major source of MSCs used for bone engineering applications [6, 7]. Compared to bone marrow, adipose tissues are easier to harvest and are considered a more practical alternative source for MSCs. Indeed, some studies used a combination of adipose tissue-derived mesenchymal stromal cells (ADSCs) and scaffolds for bone defect reconstruction [13, 14]. Moreover, scaffolds loaded with MSCs showed greater osteogenic capacity than the scaffold alone in a large animal model [15, 16].

In this chapter, we describe a protocol that uses three-dimensional (3D) scaffolds seeded with MSCs derived from either the bone marrow or from the adipose tissue of a rabbit to reconstruct large mandibular bone defects. The rabbit is a preferred animal model for studying mandibular bone repair because rabbits return to normal function within a few days of surgery, have mandibles large enough for creating critical-size defects, and are relatively small for housing and handling [5]. This protocol also describes a modified method for seeding a high density of cells into scaffolds. It has been suggested that cell seeding density in the scaffold is critical for bone engineering [17], and a higher cell number promotes a higher cell proliferation rate and osteogenic differentiation potential [18]. Therefore, we combined three major cell seeding systems together (low-pressure system, pipette system, and syringe system) to achieve a higher cell seeding density by efficiently removing the air bubble entrapped inside the scaffold. In addition, this chapter includes the surgical steps for creating critical-size mandibular defects, scaffold implantation, and micro-computed tomography (micro-CT) analysis.

2 Materials

2.1 Animals

1. 6–8 weeks old New Zealand male rabbits can be used for the isolation of bone marrow-derived mesenchymal stromal cells (BMMSC) and adipose tissue-derived stem cells (ADSC).
2. Adult New Zealand female rabbits (age: 12 months old; weight: 2.5–3.5 kg) can be used as recipient animals. All animals are kept under clean condition and provided with food and water in the animal resource center.

2.2 Isolation and Culture of Bone Marrow Mesenchymal Stromal Cells (BMMSC)

1. Sodium pentobarbital.
2. 70% ethanol in distilled water.
3. Sterile surgical instruments including sharp straight scissors, forceps, and scalpels.
4. Washing buffer: Phosphate-Buffered Saline (PBS) with 5% antibiotic-antimycotic.
5. Flushing buffer: alpha Minimum Essential Medium (α-MEM) with 2% antibiotic-antimycotic.
6. 100 mm Tissue culture dish.
7. 2 mL, 5 mL, and 10 mL sterile pipettes.
8. Pipette-aid.
9. 5 mL syringe and 21G needle.
10. 70 μm cell strainer.
11. 50 mL conical tube.
12. Trypan blue stain 0.4%.
13. Neubauer counting chamber.
14. T-75 tissue culture flasks.
15. Complete culture medium (growth medium): alpha Minimum Essential Medium (α-MEM), 10% FBS, 1% antibiotic-antimycotic, 1% L-Glutamine.

2.3 Isolation and Culture of Adipose Tissue-Derived Stem Cells (ADSC)

1. Sterile surgical instruments including sharp straight scissors, forceps, and scalpels.
2. Washing buffer: Phosphate-Buffered Saline (PBS) with 5% antibiotic-antimycotic.
3. Digestive buffer: 0.075% Collagenase type I in PBS with 2% antibiotic-antimycotic.
4. Red Blood Cell Lysis Buffer.
5. 100 mm tissue culture dish.
6. 70 μm cell strainer.

7. 50 mL conical tube.
8. Complete culture medium (growth medium): alpha Minimum Essential Medium (α-MEM), 10% FBS, 1% antibiotic-antimycotic, 1% L-Glutamine.

2.4 Multilineage Differentiation of BMMSC and ADSC

2.4.1 Osteogenic Differentiation

1. Osteoblast differentiation medium: α-MEM, 1% antibiotic-antimycotic (100 U/mL penicillin-G, 100 μg/mL streptomycin, and 0.25 μg/mL Amphotericin B), supplemented with 10% FBS, 0.1 mM ascorbic acid, and 10^{-8} M dexamethasone, 2 mM β-glycerophosphate.
2. Alizarin Red S solution: 1% Alizarin red S in distilled water.
3. 70% ethanol.
4. PBS.
5. Distilled water.

2.4.2 Adipogenic Differentiation

1. Adipogenic differentiation medium: α-MEM, 1% antibiotic-antimycotic, 10^{-8} M dexamethasone, 10 μg/mL insulin, 0.5 mM 1-Methyl- 3–Isobutylxanthine (IBMX), 0.5 μM hydrocortisone, 60 μM Indomethacin.
2. Oil Red O stain: 0.3% oil red O staining solution. 0.3 g oil red O (ICN Biomedicals) stain dissolved in 100 mL isopropanol.
3. PBS.
4. 10% Neutral Buffered Formalin.
5. 60% Isopropanol.
6. Distilled water.

2.4.3 Chondrogenic Differentiation

1. 15 mL conical tubes.
2. Chondrogenic differentiation medium: DMEM (4.5 g/L Glucose), 1% antibiotic-antimycotic, 10% ITS + Premix Tissue Culture Supplement, 10^{-7} dexamethasone, 1 μM ascorbate-2-phosphate, 1% sodium pyruvate, and 10 ng/mL transforming growth factor-beta 1 (TGF-β1).
3. Anti-Collagen II antibody.

2.5 Cell Seeding on Three-Dimensional (3D) Scaffold

1. 12-well plate.
2. 0.25% Trypsin-EDTA.
3. Three-dimensional (3D) scaffold.
4. 60 mL syringe.
5. Sterilized tweezers.
6. Air pump.

2.6 Transplantation Surgery

1. Sterile and sanitized surgical area.
2. Hand washing area.

3. Surgical attire: clean scrubs, masks, bonnets, sterile gloves/gowns.
4. Instrument sterilizer, adequate ventilation hood.
5. Buprenorphine 0.05 mg/kg subcutaneous, ketamine 20–25 mg/kg intramuscular and fentanyl 12.5 mcg/hour transdermal patch as analgesic (*see* **Note 1**).
6. Isoflurane 2% inhalant, Xylazine 5 mg/kg intramuscular and Acepromazine 0.75 mg/kg intramuscular for anesthetic.
7. Cefazolin 12 mg/kg intravenous as antibiotic.
8. 1% Xylocaine with epinephrine used as local anesthesia.
9. 20–27 gauge needle.
10. Animal restraint and tissue retraction systems adaptable to animal size.
11. External heat source(s) (e.g., Recirculating water blanket, microwaveable heating packs, or self-regulating heating pad).
12. Ophthalmic ointment (lubricant).
13. Topical antiseptic soap, sterile saline, water and/or 70% ethanol.
14. Hair removal blade, shaver.
15. Initial incision: Surgical blade (#11, #15).
16. Monopolar cautery.
17. Clamp or dissector (e.g., Mosquito clamp, McCabe facial nerve dissector).
18. Forceps.
19. Round diamond bur size#4 with high-speed handpiece.
20. Irrigation with saline.
21. Needle driver (e.g., Mayo-Heagar, Crile-Wood, etc.).
22. Absorbable suture material (e.g., 4-0 Vicryl, 5-0 Monocryl).
23. Scissors.
24. Sterile, clean cages for post-surgery recovery.
25. Tissue harvesting: scissors, low-speed engine.
26. Micro-computed tomography (micro-CT) for 3D analysis.

2.7 Micro-Computer Tomography (Micro-CT) Analysis

1. Micro-CT scanner (*see* **Note 2**).
2. Computing equipment for image reconstruction.
3. Sample holders (e.g., cylindrical vials from the manufacturer, polystyrene tubes, pipette tips, styrofoams).
4. 4% formalin in phosphate-buffered saline (PBS).
5. 70% ethanol.
6. Parafilm® or any other plastic material not containing chloride (if scanning is performed in air).

3 Methods

3.1 Isolation and Selection of Bone Marrow-Derived Mesenchymal Stromal Cells (BMMSC)

1. Euthanize rabbits by an overdose of Sodium Pentobarbital (*see* **Note 3**).
2. Shave and wash legs of rabbit by 70% ethanol.
3. Incise and peel skin to expose the hind limb. Use sterile sharp scissors to cut the joints and remove the muscles and ligaments.
4. Remove femur and tibia at the knee and ankle joints and place in cold washing buffer (PBS with 5% anti-anti) (*see* **Note 4**).
5. Wash bones for 5 min × 3 times in washing buffer.
6. Cut the ends of bones to expose the bone marrow. Flush out the marrow plug with a 21G needle attached to a 5 mL syringe filled with flushing buffer.
7. Drawing flushing buffer and marrow plugs up and down several times to make a single cell suspension.
8. Transfer cell suspension through a 70 μm cell strainer placed on the top of a 50 mL conical tube.
9. Centrifuge at 300 × *g* for 5 min at 4 °C and discard the supernatant. Cell pellet is resuspended in the complete culture medium.
10. 50×10^6 cells are seeded in a T-75 cell culture flask and incubated at 37 °C in a 5% humidified incubator.
11. After 3 days, remove floating cells by washing with PBS and add fresh culture medium. Change half of the medium every 2–3 days until the cells get to a 70–80% confluency.

3.2 Isolation of Adipose Tissue-Derived Stem Cells (ADSC)

1. Euthanize rabbits by an overdose of Sodium Pentobarbital (*see* **Note 3**).
2. Shave and wash inguinal region, neck and back region of rabbit by 70% ethanol.
3. After incising the skin, subcutaneous adipose tissues at inguinal region and neck and back region are removed and put in cold washing buffer.
4. Wash tissues for 5 min × 3 times in washing buffer.
5. After removing the debris, adipose tissues are placed in the tissue culture dish with around 2 mL digestive buffer.
6. Mince the tissues into small pieces with sterile sharp scissors and pipette up and down several times with a 25 mL pipette to further facilitate the digestion.
7. Transfer tissues to a new 50 mL conical tube, add more digestive buffer (1:1, buffer: adipose tissue).
8. Incubate the tissue on a shaker for 30 min at 37 °C in a 5% humidified incubator.

9. Neutralize the collagenase type I with the same amount of alpha MEM containing 20% FBS.
10. Shake the tube vigorously several times to further disintegrate the aggregate of adipose tissue.
11. Centrifuge the sample for 5 min at 800 × *g*, 4 °C.
12. Take out the sample from the centrifuge and shake it vigorously to disrupt the cell pellet. Repeat the centrifugation step.
13. Pour out (discard) the adipocytes layer and supernatant containing the collagenase type I without disturbing the cell pellet.
14. Resuspend the cell pellet in 1 mL RBC lysis buffer, and incubate for 10 min on top of ice.
15. Wash with 20 mL of PBS with 2% antibiotics-antimycotics and centrifuge at 800 × *g* for 5 min.
16. Discard the supernatant and resuspend the cells in complete culture medium.
17. Filter cell suspension through a 70 μm cell strainer. Wash the cell strainer with additional 2 mL culture medium to obtain any additional cells.
18. Seed cells in a proper tissue culture plate and incubate at 37 °C in a 5% humidified incubator.
19. After 3 days, floating cells are removed by washing with PBS and add fresh culture medium. Change half of the culture medium every 2–3 days until the cells reach a 70–80% confluency.

3.3 Multilineage Differentiation of BMMSC and ADSC

3.3.1 Osteogenic Differentiation

1. Seed cells in 6-well plate with growth medium and incubate at 37 °C until they reach approximately 50–70%.
2. Aspirate the growth medium and replace with 2 mL of osteogenic differentiation medium per well.
3. Incubate the cells at 37 °C in a 5% humidified incubator and change medium every 2–3 days.
4. After 3 weeks induction, osteogenic differentiation is visualized by Alizarin Red S staining (*see* Fig. 1b).

3.3.2 Adipogenic Differentiation

1. Cells are seeded in 6-well plate with growth medium and incubated at 37 °C until they reach approximately 90–100% confluency. It takes approximately 1–4 days.
2. Aspirate the growth medium and replace with 2 mL of adipogenic differentiation medium per well.
3. Incubate the cells at 37 °C in a 5% humidified incubator and change medium every 2–3 days.
4. After 3 weeks induction, adipogenic differentiation is visualized by Oil Red O staining (*see* Fig. 1a).

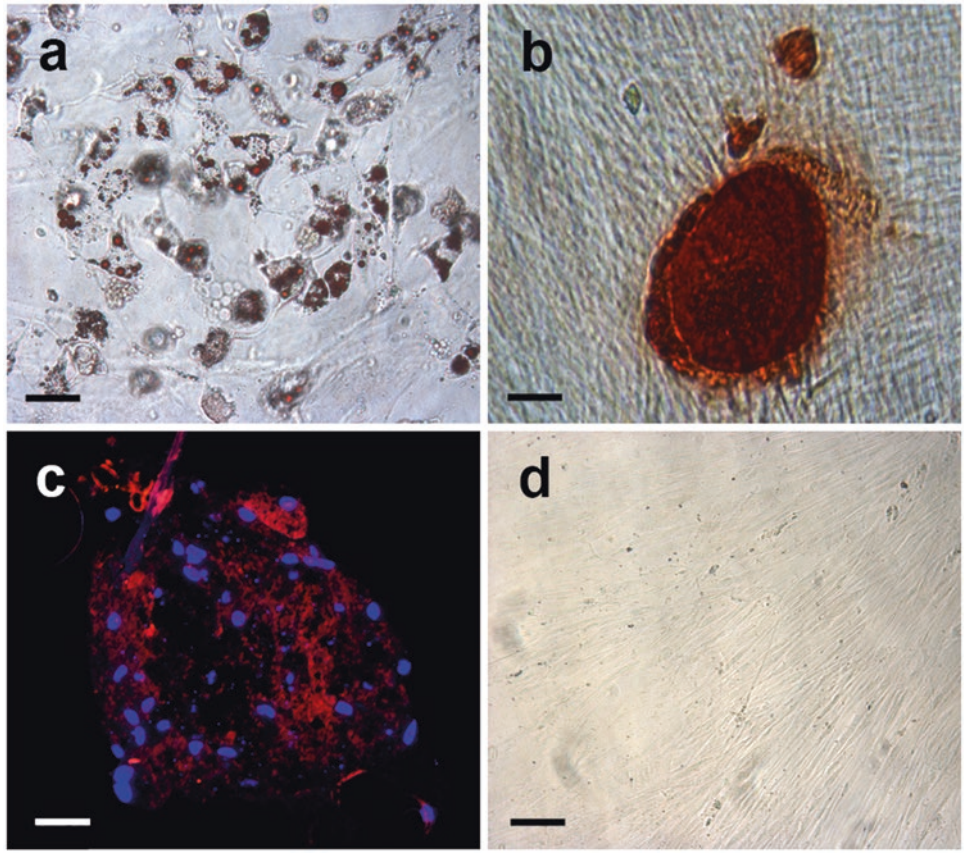

Fig. 1 Multilineage differentiation of rabbit adipose tissue-derived stem cell (ADSC). (**a**) Oil red staining for adipogenic differentiation. (**b**) Alizarin Red staining for osteogenic differentiation. (**c**) Collagen type II immunofluorescent staining (in *red*) for chondrogenic differentiation; cell nuclei are stained in *blue*. (**d**) Rabbit ADSCs with growth medium. Scale bar = 38 μm

3.3.3 Chondrogenic Differentiation

1. 5×10^5 cells are resuspended with 5 mL growth medium in a 15-mL conical tube.
2. Centrifuge the cells at 200 × *g* for 5 min at room temperature. Discard the supernatant and resuspend the cells with 0.5 mL chondrogenic differentiation medium.
3. Centrifuge the cells at 200 × *g* for 5 min at room temperature. Do not remove the medium. Loose the cap of the tube to allow gas exchange, incubate upright at 37 °C in a 5% humidified incubator (*see* **Note 5**).
4. Change medium carefully every 2–3 days. Ensure the cell ball is released from the wall of tube and float freely (*see* **Note 6**).
5. Chondrogenic cell pellets are harvested after 14–28 days in culture.
6. Cell pellets are cryopreserved and sectioned into 5–8 μm.
7. Chondrogenic differentiation is assessed by immunofluorescent staining for collagen type II (*see* Fig. 1c).

3.4 High Cell Density Seeding on 3-D Scaffold (See Fig. 2)

1. Aspirate all cell growth medium and wash the cell monolayer twice with 37 °C PBS to remove any residual FBS (*see* **Note 7**).
2. Add enough pre-warmed 0.25% Trypsin-EDTA to cover the cell layer.
3. Incubate the cells for 2 min at 37 °C. Tap the bottom gently to dislodge the cells.
4. Add the same amount of complete culture medium to neutralize the Trypsin. Gently rinse the cell layer several times with a pipette to detach all cells.
5. Transfer the cell solution to a new 15 mL or 50 mL conical tube.
6. Centrifuge the cells at 300 × *g* for 5 min at 4 °C. Remove the supernatant and resuspend the cell pellet in complete culture medium.
7. Place autoclaved scaffolds in a 60 mL syringe and aspirate 20–30 mL complete culture medium.
8. Connect syringe to the air pump.
9. Turn on the pump and hold the syringe plunger to create a negative pressure.
10. Tap the syringe barrel gently to free the air bubbles trapped in the scaffold.
11. Transfer scaffolds to 12-well plate with sterile tweezer.
12. 2.5×10^6 cells are seeded on the scaffold and incubated at 37 °C in a 5% humidified incubator (*see* **Note 8**).
13. After 3–5 days, scaffolds with cells are ready for the transplantation.

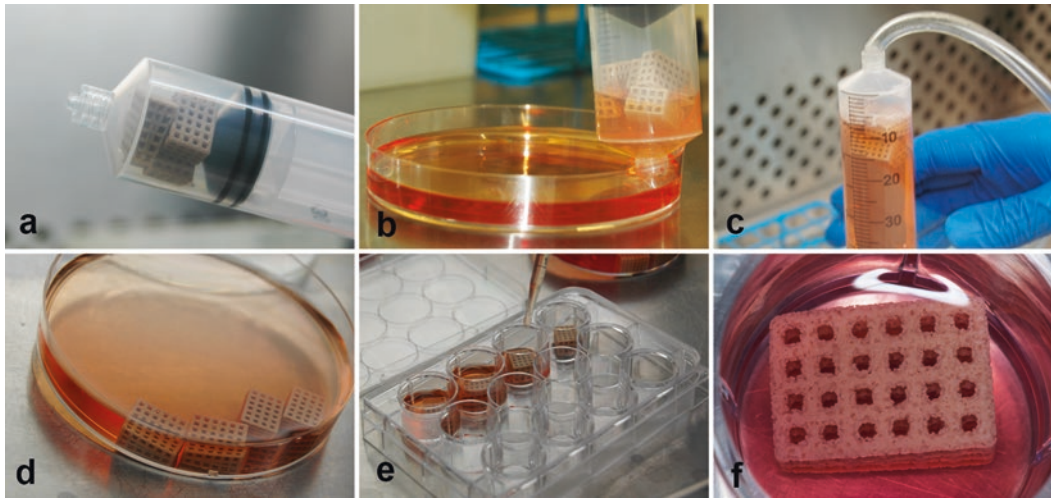

Fig. 2 Procedures for cell seeding on 3-D scaffolds. (**a** and **b**) Place autoclaved scaffolds in a 60 mL syringe and aspirate 20–30 mL of complete culture medium. (**c**) Connect syringe to the air pump to remove the air bubbles entrapped in the scaffold. (**d**) Transfer scaffolds to a 12-well plate with sterile tweezer. (**e** and **f**) 2.5×10^6 cells are seeded on each scaffold and incubated at 37 °C in a 5% humidified incubator

3.5 Transplantation Surgery

3.5.1 Preoperative Surgery Preparation

1. Rabbit acclimation of 7–14 days in the animal center is strongly recommended for their maximal adjustment prior to the surgery.
2. Place animals in appropriate housing soon after their arrival.
3. Surgeon and surgical assistants wear clean scrubs, shoe covers, masks, bonnets with sterile gown/gloves.
4. Observers wear clean scrubs, show covers, masks, and bonnets.
5. All instruments are sterilized prior to surgery.
6. Clean instruments before sterilization to remove organic material.
7. Wrap instruments in peel packs, include sterilization indicator.
8. Achieve sterilization by autoclaving (steam), or gas sterilization with ethylene oxide.

3.5.2 Anesthesia, Intubation, and Antiseptic Preparation

1. Animals anesthetized in area designated for surgical preparation.
2. Anesthetize the animal: Buprenorphine 0.05 mg/kg given subcutaneously 30 min preoperatively. Xylazine-Acepromazine 5 mg/kg, and 0.75 mg/kg given intramuscularly during induction.
3. Anesthesia maintained with Isoflurane 2% inhaled via the endotracheal tube.
4. Place animals in prone position with neck extended and mouth facing upward.
5. 30 mm I.D. endotracheal tube is placed in mouth and advanced until vapor from lungs is observed in tube.
6. Once endotracheal tube is placed, auscultation of both lung bases is performed to confirm proper tube placement.
7. Fix tube at 10 cm to the mouth using cling wrapped around the nape of the neck.
8. Animal is placed in supine position with a shoulder roll (i.e., wrapped sterile towel).
9. Sterile ophthalmic ointment is applied to both eyes to prevent corneal desiccation and abrasion.
10. Shave the surgical site twice the size of the expected field with an electric razor. Remove all loose hair and debris from the animal using tape.
11. Antiseptic skin preparation: Use aseptic technique when performing skin antisepsis. Start at the center of the site and move in a circular motion outward. Perform three scrubs using an antiseptic soap and gauze (e.g., povidone-iodine solution or 2% chlorhexidine solution).

12. Sterile surgical draping: Drape the animal with a sterile, impermeable covering to isolate the disinfected area. Fix the drape in place with tape or clamps. Cover a stand with sterile drape for placement of sterile instruments.
13. Confirm depth of anesthesia before operating with pedal withdrawal reflex and vital signs.

3.5.3 Surgical Procedures and Monitoring

1. Maintain aseptic conditions during all procedures.
2. Continuously monitor heart rate and rhythm, blood pressure, respiratory rate and depth and temperature and document every 10 min.
3. Inject locally 2–3 mL of 1% Xylocaine with epinephrine. The injection depth is to the area of the planned incision and dissection.
4. 2 cm incision made superficially in the skin over the inferior border of the mandible (*see* Fig. 3b).
5. Dissection to the level of the mandible (*see* Fig. 3c, d, and e). Combination of monopolar cautery and careful dissection

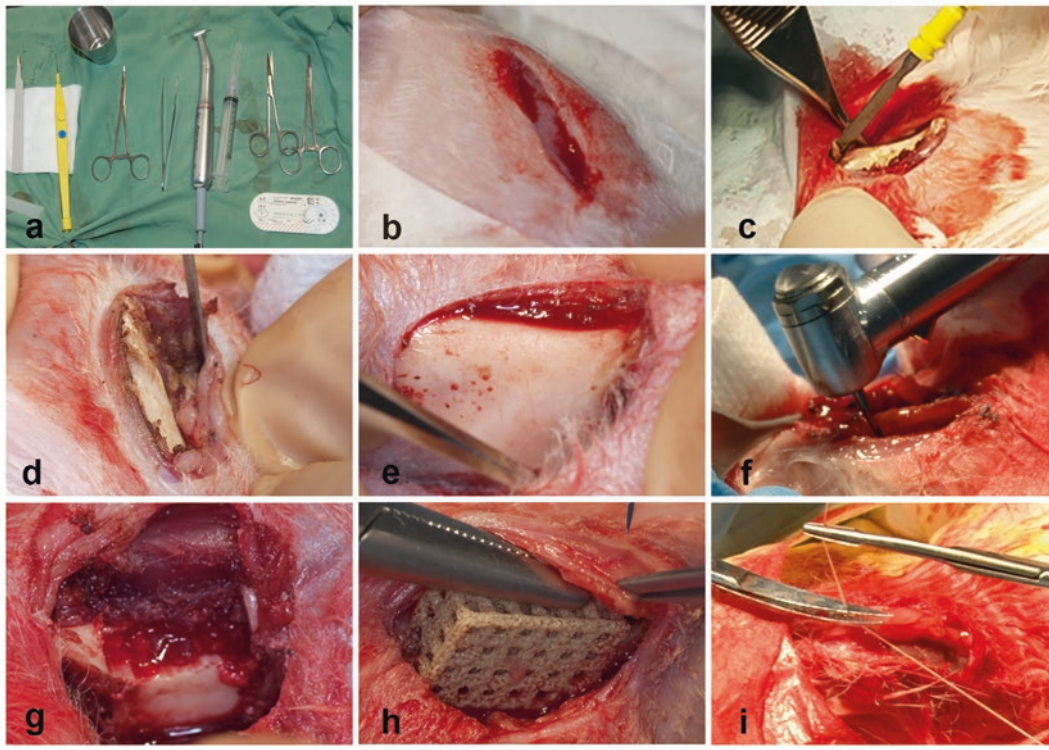

Fig. 3 Procedures for creating a critical size bone defect on rabbit mandibles and transplantation of the scaffold seeded with MSC. (**a**) Surgical instruments. (**b**) 2 cm incision made superficially in the skin over the inferior border of the mandible. (**c**, **d,** and **e**) Dissection to the level of the mandible. (**f** and **g**) Critical size defect created (1.5 cm*1.0 cm). (**h**) Insert and stabilize the scaffold with resorbable sutures to surrounding soft tissues. (**i**) Reposition the muscle and parotid gland using resorbable sutures

using clamp and forceps. Dissect through the masseter muscle at the pterygomasseteric sling. Expose body of the mandible using blunt dissection in a subperiosteal plane.

6. Critical size defect created (*see* Fig. 3f and g): Target defect creation in body of mandible inferior to the tooth roots. Initiate access with round diamond bur size #4 to outline 1.5 cm by 1.0 cm rectangular marginal defect at the inferior border of the mandible. Complete the bicortical defect using the same bur.
7. Insert and stabilize scaffold with resorbable suture to surrounding soft tissue (*see* Fig. 3h).
8. Closure: Reposition the muscle and parotid gland using resorbable suture (i.e., 4-0 Vicryl) (*see* Fig. 3i). Reapproximate the skin using a 5-0 Monocryl suture in a running subcuticular fashion.

3.5.4 Postoperative Care

1. Place animals in a clean, quiet environment for anesthetic recovery until they can maintain a patent airway and sternal recumbency.
2. Keep animal in a warm and dry environment with water circulated heating pad, air circulating heating blanket, or surgical thermal barrier.
3. Administer analgesics post-surgically and for the next 72 h. Buprenorphine 0.02–0.05 mg/kg subcutaneously every 8–12 h. Avoid non-steroidal anti-inflammatory drugs that may inhibit bone formation.
4. Examine the wound daily until completely healed. It usually takes 3 to 5 days for the wound healing of skin.
5. Monitor weights daily for the first week and assess hydration status clinically (e.g., Energy, vital signs).

3.6 Micro-CT Analysis (*See* Note 9)

Micro-CT involves obtaining a sequence of X-ray images of a particular sample at different rotations, and then using computer algorithms to reconstruct a 3D image. The micro-CT procedures can be divided into four general steps:

1. Specimen preparation before data acquisition.
2. Acquiring the X-ray projection images.
3. Computerized reconstruction of 3D of images from the projection images.
4. Analysis of the 3D image stack.

3.6.1 Sample Preparation before Data Acquisition

Specimens from many species can be analyzed using micro-CT; however, the examples here are based on the analysis of rabbit mandibles with implanted biomaterials. In several studies, the

researchers want to perform histological evaluation on the same samples after micro-CT scanning. If this is the case, bone samples need to be fixed overnight in 4% buffered formalin after dissection, then washed with PBS and stored in 70% ethanol at 4 °C before scanning.

3.6.2 Acquiring the X-ray Projection Images (Scanning)

The first step is to acquire the X-ray images to reconstruct the region of interest. This step is usually referred to as "scanning."

1. Switch on the SkyScan 1172 instrument and the micro-CT computer(s), and open the SkyScan software.
2. Turn on the X-ray source and allow it to run for 10–15 min to stabilize the X-ray beam.
3. Set up the scanning parameters (e.g., voltage, resolution, filtering, etc.). The optimal settings depend on the material type, object thickness, scanning medium (air or fluid) and on what needs to be analyzed (*see* Table 1**, Note 10**).
4. Perform a flat-field correction to correctly calibrate the scanner for the background readings (*see* **Note 11**).
5. Prepare specimen for scanning by removing it from the fixation or storage medium and wrapping them with a Parafilm® to prevent specimen drying (*see* **Note 12**).
6. Mount the specimen in an appropriate holder that is relatively transparent to the X-ray beam (e.g., cylindrical vials from the manufacturer, polystyrene tubes, pipette tips, styrofoams) (*see* **Note 13**).
7. It is preferable that the long axis of the sample is to be aligned with the rotation axis of the scanner to reduce beam hardening and obtain the best image quality. Once the sample is loaded in the scanner, a scout scan is performed to set up the appropriate sample position and area of interest. Then, start the scan.

Table 1
Suggested parameters for the scan of rabbit bone specimens using the SkyScan1172 instruments

X-ray voltage	50 kV
X-ray current	200 μA
Filter	0.5 mm aluminum
Camera resolution	Medium
Pixel size	10–15 μm
Tomographic rotation	360°
Rotation step	0.3–0.5
Frame averaging	2–4

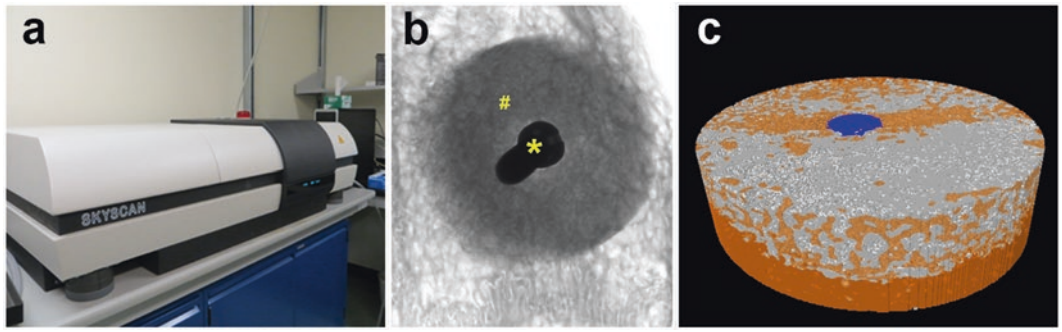

Fig. 4 Micro-CT analysis. (**a**) SkyScan 1172 micro-CT scanner. (**b**) 2D X-ray projection showing three materials with different X-ray attenuations: titanium screw (*), biomaterial (**#**), and rabbit bone. (**c**) 3D reconstructed micro-CT image using CTAn software showing three materials demonstrating a titanium screw (*blue*), the biomaterial (*orange*), and the rabbit bone (*light gray*)

Table 2
Image reconstruction parameters

Beam hardening correction	20%
Ring artifact correction	10
Smoothing	0
Misalignment compensation	Varied (−0.5 to 1.5)

3.6.3 Image Reconstruction (See Fig. 4)

1. After the scan is complete, load the raw image dataset in the NRecon software and select the part of the scan to be reconstructed (avoid including images areas outside the sample to decrease the dataset size and reconstruction time).
2. Set the reconstruction parameters; beam hardening correction, ring artifact correction, smoothing, and misalignment compensation. The optimal settings need to be empirically evaluated depending on the type of scanner, sample, and scanning parameters (*see* Table 2**, Note 14**).
3. After selecting the parameters, click on the preview of a single slice to determine whether the settings are correct. Fine-tuning option runs a series of previews by adjusting one parameter or several parameters at the same time to select the most optimal setting.
4. Select the data dynamic range from the histogram and select the appropriate image file format (*see* **Note 15**).
5. Create a new folder from the "raw dataset" folder to save the reconstructed images and run the reconstruction. In case you want to reconstruct more than one sample at the same time, select the "add to batch" option and run the reconstruction after adding the final sample.

3.6.4 Analysis of the 3D Image Stack (See **Note 16***)*

1. Upon opening the dataset in CTAn (software provided by SkySkan), select a region of interest (ROI) containing the bone volume that needs to be analyzed around the implanted biomaterials (*see* **Note 17**).
2. Proceed to the binary selection page and choose a threshold range that selects structures to be analyzed, in this case bone, based on gray scale values.
3. Proceed to custom processing and run the thresholding using the selected values. Use despeckling to remove any possible noise "white dots or speckles."
4. Finally, the 3D analysis plug-in can be run to calculate the bone volume within the selected ROI. Several other parameters can be calculated at the same time (e.g., porosity, structure thickness, etc.).

4 Notes

1. Drugs used will vary according to veterinary instructions.
2. The SkyScan systems are designed to work mainly with standard Microsoft Windows®-based computers.
3. Animals should be sacrificed using procedures approved by animal facility.
4. From this step, the bones are transferred to the tissue culture laboratory for bone marrow harvesting.
5. After 1–2 days, cell pellet forms a round ball. The pellet remains the same size for the entire culture time.
6. Medium are removed carefully to avoid aspirating the pellet.
7. Alternatively, Ca^{2+}- and Mg^{2+}-free HBSS can be used for washing. Cells at passage 3–6 are used for transplantation.
8. Scaffold should be fully immersed in the culture medium.
9. Microcomputed tomography (micro-CT) can be performed either on live animals (in-vivo scanning) or after extracting the specimens from animals (ex-vivo scanning). Several micro-CT systems are available for the study of bone and materials (e.g., Scano, SkyScan, XRadia, etc.). Micro-CT measurements vary according to the scanned sample, the scanner used, and what needs evaluation. Therefore, this section highlights the points to be considered for micro-CT measurements and focuses on scanning ex-vivo specimens using the SkyScan system.
10. These are the suggested parameters of the scan for our specimen using the SkyScan1172 instruments (Table 1).
    (a) Energy: Higher energy X-ray beam allows better penetration of high-density materials, while low energies yield

better contrast when scanning different materials. Therefore, optimal energy is a tradeoff between intensity and contrast.

(b) Filtering: To minimize the effects of beam hardening, some manufacturers provide a set of filters that can directly absorb the low-energy photons. Using filters narrows the X-ray beam spectrum and makes the images more suitable for quantitative analysis. However, filtering reduces the overall intensity which can be compensated by increasing the exposure time, thus increasing the overall scanning time.

(c) Exposure time: A longer exposure time improves the image quality by reducing noise-to-signal ratio but increases the overall scanning time. However, too high exposure time can fully saturate the micro-CT detector and yield image artifacts.

(d) Frame averaging: This option allows imaging each projection several times and using the average for image reconstruction. Similar to increased exposure time, it reduces noise-to-signal ratio, improves the image quality, and increases the overall scanning time. However, the advantage of frame averaging over increasing exposure time is that it avoids saturating the micro-CT detector.

(e) Resolution: The optimal resolution depends on the specimen size and the features to be analyzed.

(f) 180° or 360° rotation: 180° scans are used to shorten the overall scanning time, since the projection images from 0 to 180° are the mirror images of the project images from 180 to 360°. However, 360° scans are required when scanning complex structures. Moreover, 360° ensures a better quality and more accurate images.

11. Flat-field correction and alignment checks are performed immediately after the installation of the scanner, and repeated every 4 and 8 weeks afterward, respectively. However, it is recommended to run flat-field correction if the parameters of the scan are changed.

12. Other plastic films can be used that are also relatively transparent to X-rays except the ones containing chloride, since it affects the attenuation of the X-ray beam. Even though micro-CT scanning is a nondestructive method, the heat generated during scanning might dry out the specimen that is why the wrapping step is critical when scanning the specimens in air. On a side note, specimens can be measured both, in air or liquid. In case the specimens need to be measured in liquid (usually saline or 70% ethanol), then avoid creating large air bubbles by adding the liquid slowly using a syringe and then tapping the sample holder to remove any trapped bubbles. Moreover,

prevent the liquid from evaporating by closing the sample holder with the provided lid or plastic film.

13. The size of the sample holder depends on the size of the sample and how many samples you want to analyze at the same time using the batch scanning option. In order to obtain an accurate reconstruction algorithm and prevent motion artifacts, it is critical that there is no relative movement between the specimen and the sample holder during scanning. Therefore, ensure samples fit tightly inside the sample holder by using addition wrapping film, if necessary, without applying too much force to avoid breaking your specimen. Use the most appropriate holder size to avoid using too many wrapping films.
14. We used the following settings in our reconstructions (*see* Table 2)
    (a) Beam hardening is a micro-CT artifact that results due to the fact that the X-ray beam produced by the micro-CT scanner is not composed of single energy X-rays, but rather a spectrum of X-rays. When the X-ray beam hits the sample, the lowest X-ray energies are absorbed first by the outer layers of the samples, while the remaining higher X-rays pass through the rest of the sample. This makes the outer layers of the sample appear as if they have higher X-ray attenuation. The beam hardening correction parameter tries to correct this inherent artifact.
    (b) Ring artifacts are common artifacts appearing as rings or half-rings in the reconstructed images, attributable to a defect in the scintillator that converts X-ray to visible light, or simply to dust on the detector system. Ring artifact correction tries to replace these artifacts by averaging the neighboring pixels. A higher ring reduction means a more precise reconstruction but increased reconstruction time.
    (c) Smoothing produces 3D images with less noise; however, it reduces the ability to detect fine details in the sample. Therefore, it is recommended to avoid this option if a precise analysis is required.
    (d) Misalignment compensation improves the accuracy of reconstruction by compensating for any possible misalignment during acquisition. Misalignment can differ from sample to sample; however, to compare different samples the other parameters should be the same.
15. This will scale the raw image data set to either 8-bit integer or 16-bit integer image file. Use the same option for all samples in the experiment. (We usually select the JPG file format).
16. Several outcomes can be evaluated from the 3D images (e.g., bone volume, biomaterial volume, bone mineral density, porosity, pore size, etc.). It is important to establish a

standardized analysis method to be applied to all samples and can be repeated by any user without any bias. The analysis section mentioned here is designed to highlight the steps for measuring bone volume around an implanted biomaterial that has a different X-ray attenuation from the measured bone.

17. To standardize the analysis, use the same ROI shape and size for all samples and with the biomaterial being centered inside the selected ROI. This is why it is important to scan all samples in the same orientation during image acquisition.

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Author's Contribution

This study was designed by D.F., M.R., M.N.A., F.T., S.T. D.F., J.J. conducted the isolation and characterization of MSCs. D.F., M.R., and M.B. conducted the transplantation of scaffolds. MNA contributed to the scaffold analysis and micro-CT scan. D.F., M.R., M.N.A., M.B., L-C.L., and S.T. wrote and revised the manuscript. S.T. supervised this study and directed final version of all contents. All authors reviewed and approved the manuscript.

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Chapter 23

Optimal Environmental Stiffness for Stem Cell Mediated Ischemic Myocardium Repair

Honghai Liu, Christian Paul, and Meifeng Xu

Abstract

Cardiovascular diseases related to myocardial infarction (MI) contribute significantly to morbidity and mortality worldwide. The loss of cardiomyocytes during MI is a key factor in the impairment of cardiac-pump functions. Employing cell transplantation has shown great potential as a therapeutic approach in regenerating ischemic myocardium. Several studies have suggested that the therapeutic effects of stem cells vary based on the timing of cell administration. It has been clearly established that the myocardium post-infarction experiences a time-dependent stiffness change, and many studies have highlighted the importance of stiffness (elasticity) of microenvironment on modulating the fate and function of stem cells. Therefore, this chapter outlines our studies and other experiments designed to establish the optimal stiffness of microenvironment that maximizes benefits for maintaining cell survival, promoting phenotypic plasticity, and improving functional specification of the engrafted stem cells.

Key words Myocardial infarction, Stem cell therapy, Stiffness of microenvironment, Hydrogel

1 Introduction

Cardiovascular diseases related to myocardial infarction (MI) contribute significantly to morbidity and mortality worldwide despite advancements in medicine. The loss of cardiomyocytes during MI is a key factor in the impairment of cardiac-pump function due to the limited regenerative capacity of heart tissues. Using cell transplantation to regenerate ischemic myocardium has been considered as a new potential therapeutic approach to replace the lost or damaged cardiac cells. The results from experimental studies have suggested that transplanted stem cells can promote cardiac functional recovery after acute myocardial infarction (AMI) [1–4]. Mesenchymal stem cells (MSCs) are a particularly attractive option because they are both multipotent and immune privileged. There is some evidence that stem cells can improve cardiac function in patients suffering from AMI [5–8]. It has also been demonstrated that the ability of stem cells to repair the myocardium is not only dependent on the transdifferentiation of stem cells into cardiac

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phenotypes [9–11], but also on the protection of the native myocardium which is mediated primarily by paracrine factors released from stem cells [1, 3]. However, several studies have suggested that the therapeutic effects of stem cells are varied based on the timing of cell administration [12–14]. An analysis of seven trials with 660 patients indicated that bone marrow stem cells (BMSCs) transferred at 4–7 days post-AMI is superior to that within 24 h in improving cardiac function [14]. It has been observed that stem cell therapy at 1 week after AMI facilitates integration of transplanted cells and functional recovery [13]. The optimal efficacy of bone marrow stem cell therapy at 7–14 days after MI may result from non-VEGF dependent angiogenesis [15]. Cell transfer within 24 h post-AMI does not augment recovery of global left ventricular contractile function [8]. Therefore, the optimal window of opportunity for stem cell therapy for MI might range in the period from day 5 to week 2 after the infarction [13–15].

The microenvironment around transplanted cells after MI potentially plays an essential role in deciding the optimal timing of cell therapy [15]. Many studies have highlighted the importance of stiffness (elasticity) of myocardium and composition of the extracellular matrix (ECM) on modulating the fate and function of stem cells including renewal, proliferation, differentiation, and regenerative potential [16–21]. Thus, the optimal stiffness of myocardium within a certain time frame post-AMI might offer some benefits for maintaining cell survival, promoting phenotypic plasticity, and improving functional specification of the engrafted stem cells [15].

2 The Myocardium Post-infarction Experiences a Time-Dependent Stiffness Change

In most soft tissues, cells added to an ECM establish a relatively elastic microenvironment. Myocardial stiffness is an index of muscle properties and is important in understanding normal and abnormal physiology [22]. Titin is a large elastic protein that extends across each half-sarcomere and is stretched in diastole when the sarcomere relaxes. However, at long sarcomere lengths or in damaged cardiomyocytes, collagen in the ECM increasingly contributes to stiffness and changes in a time-dependent manner from flexible to rigid following MI [23]. This translates into myocardial loss, subsequent remodeling, progressive ventricular dilatation, and fibrosis. Cardiomyocytes first undergo irreversible cell death, inducing an acute inflammatory reaction in the ischemic myocardium. Neutrophils and macrophages then quickly infiltrate the infarct region and release inflammatory mediators and matrix metalloproteinases (MMPs) to degrade ECM between 24 and 72 h [24]. Given that cardiomyocytes possess limited regenerative capacity, the spared myocardium becomes composed of the surviving hypertrophic cardiomyocytes as well as remodeling and degradation of the surrounding ECM resulting in scar formation. Finally, a matured

collagen-rich reparative scar is formed to replace the extensive loss of cardiomyocytes in the infarct zone [25]. During this period, the proper balance between ECM synthesis and degradation is critical for optimal infarct healing. Excessive ECM accumulation increases wall stiffness and impairs compliance, leading to diastolic dysfunction [25, 26]. Atomic force microscopy has been used to map myocardial elasticity and establish the baseline elastic modulus for normal heart muscle at 18 ± 2 kPa [4]. The stiffness of infarcted myocardium between 1 to 24 h after AMI is relatively soft (4–17 kPa) [27]. Two weeks post-ischemia, infarcted myocardium formed significant fibrosis, with a similar threefold increase in the elastic modulus (55 ± 15 kPa). Injection of MSCs exhibited a significantly softer tissue modulus (40 ± 10 kPa) compared to the infarcted area in animals without MSC treatment [4]. This potentially indicates that the stiffness of infarcted myocardium may play a significant role in the post-infarction remodeling process and any intervention that softens the infarct region may reduce deleterious remodeling.

3 The Role of Environmental Stiffness on Function and Fate of Stem Cells

Successful engraftment and survival of stem cells are the most important factors when assessing the effectiveness of stem cell therapies [28]. Implantation of stem cells via intramyocardial or coronary injection is plagued by limited cell retention and survival due to the lack of ECM in the infarcted myocardium [29, 30]. Injected or transplanted cells remain at the site of treatment only for a very short duration, leading to reduced therapeutic efficacy of the transplanted cells [30–32]. Cells delivered into the heart are rapidly redistributed to the lungs, liver, spleen, other organs throughout the body, or/and cleared by the lymphatic system [33]. The infarct region is poorly oxygenated, contains dead and apoptotic cells, and has a progressively increasing stiffness, which does not support the viability and well-being of implanted cells [33]. Transplanted cells do not survive in this hostile environment in the early days after MI and most cells die within 4 days after transplantation [34, 35]. Acute retention of stem cells varies depending upon route of delivery. In an elegant study utilizing radiolabeled bone marrow mononuclear cells [36], only 11 ± 3 % of cells remained in the heart just 90 min after intramyocardial injection. The low intrinsic capacity to differentiate into cardiomyocytes may also influence their direct participation in myocardial regeneration. However, stem cells are able to feel and respond to the mechanical rigidity of a matrix changes in their mechanical environment [37, 38]. Cameron et al. [39] have recently reported that loss of rigidity has an influence on differentiation and proliferation of stem cells. Generally, soft matrices promoted significantly more proliferation and chondrogenic differentiation, whereas hard matrices promoted osteogenic differentiation [40]. The differentiation of MSCs into a specific

lineage is enhanced on a matrix designed to create stiffness that is similar to the targeted tissue type [41]. It has also been reported that soft matrices (0.1–1.0 kPa) (which mimic brain tissue) have neurogenic and stiffer matrices (11 kPa) which mimic striated myogenic muscle. Comparatively rigid matrices (34 kPa) which are heavily cross-linked with collagen more commonly initiate bone growth [42]. Stem cells cultured in a medium with a matrix stiffness (31 kPa) similar to the elasticity of infarcted myocardium at day 7 had a greater ability to differentiate into endothelial lineage cells. However, those cells grown in the medium with a relatively soft matrix (4–17 kPa) showed minimal differentiation [27]. The differentiation of ES cells into cardiomyocyte was very limited when cells were cultured on the hydrogel with stiffness similar to that of brain or fat tissue (0.2 kPa) [38].

Microenvironment stiffness also influences the proliferation of stem cells. The percentage of actively proliferating cardiomyoblasts falls from 23 % to 1 % between embryonic day 14.5 and neonatal day 7 [43], which coincides with the rise in myocardial stiffness during embryological development [44]. In addition, the marked reduction in proliferative capacity of cardiomyoblasts in vivo between embryonic day 14.5 and neonatal day 7 is surprisingly similar with that of a cell cycle altered by the gel stiffness in vitro [45].

The environmental stiffness not only influences the survival, proliferation, and differentiation of stem cells, but also influences the function of cells. A series of flexible substrates of hydrogels with stiffness similar to that of embryonic, healthy, ischemic, or fibrotic myocardium have been used to test the effects of stiffness on the contractile output of cardiomyocytes [46]. The matrices with stiffness in the range of 11–17 kPa of the developing myocardial microenvironment were optimal for promoting actomyosin striation and cardiomyocyte beating. The scar-like stiff substrate (35–70 kPa) region that mimicked a post-infarct fibrotic scar lacked striated myofibrils and stopped beating. However, on very soft matrices, cells preserved contractile beating for several days but did very little work [46]. The mechanical output has also been demonstrated using cardiomyocytes differentiated from human pluripotent stem cells that it was the highest on hydrogels with stiffness similar to that of healthy myocardium [47].

4 Hydrogel with Optimized Stiffness Improves Stem Cell Efficacy for Heart Repair

4.1 Natural and Synthetic Hydrogel

A variety of natural and synthetically formulated materials have been utilized to determine the effects of stiffness on stem cell mediated heart repair. The natural biomaterial is consisted of a three-dimensional decellularized cardiac ECM which is composed of a network of interstitial collagens. Cardiac ECM demonstrated excellent biological properties for cellular recognition, biocompatibility, and had the potential to degrade through known metabolic

processes [48–50]. These experiments were initially designed as a protective platform for cellular therapy because they can provide many benefits in maintaining the appropriate phenotypic and functional characteristics of cells [51, 52]. Cardiac ECM may improve the survival of transplanted cells via providing a suitable scaffold that structurally and biochemically mimics the native cardiac environment [52]. These ECM also provide many heparin-binding sites that enhance the loading and release of soluble growth factors [53]. A range of stiffness of natural ECM-protein-derived hydrogels are achieved by changing the density of these proteins and altering the surface ligand concentration [54].

Synthetic materials have several advantages in manufacturability and are typically inexpensive and easily reproducible. Synthetic polymer gels can serve as ECM mimetic scaffolds for stem cell fate control [42, 55, 56]. The stiffness of polymer gels can be adjusted by altering the amount of a cross-linker. Polyacrylamide (PA) is critical for biomedical applications because it is hydrophilic and bioinert. Because of the toxicity of its monomer, acrylamide [48, 57], it is necessary to characterize their biocompatibility and mechanical properties before being used in vivo [58]. Fibrin glue is a hydrogel that is widely used in surgery, cell culture, and tissue engineering. Both autologous and recombinant off-the-shelf fibrin glues have been extensively used as pro-coagulants during cardiac and other surgeries. Additionally, its angiogenic role following myocardial ischemia makes this an attractive system for possible catheter-based cell therapy of the heart [59]. A semisynthetic hydrogel has also previously been used for cardiac and skeletal muscle regeneration [60]. This type of hydrogel has a distinct advantage over other types of scaffolds because its mechanical properties are highly malleable while leaving the functionality of the encapsulated cells well preserved by the backbone of the polymeric network.

4.2 Hydrogel Improves the Function of Stem Cells

Biocompatible hydrogels have been considered in situ as a cell delivery vehicle to improve cell retention, survival, and function following delivery into the ischemic myocardium. The potential of injectable biomaterials for the delivery of stem cells and their roles in myocardium regeneration have been reported previously. Intra-myocardial polymerization of polymers in situ represents one possible solution to address both leakage and clearance-mediated cell loss [33].

A tissue-engineered, hydrogel-based endothelial progenitor cell-mediated therapy can enhance cell delivery, cell retention, vasculogenesis, and preservation of myocardial structure and function [61, 62]. Revascularization and hemodynamic parameters of infarcted heart are significantly improved by injection of cells into the infarct region of optimal stiffness of the PEG-fibrinogen (PF) scaffold [60]. The functional integration of transplanted cells and host myocardium in the ischemic heart is significantly improved [60].

The phenotype, cell proliferation, and viability of cardiomyocytes are well maintained and are higher in cells cultured with ECM than that of cells cultured without the ECM sheets. It has thus been suggested that natural ECM sheets could be used in the future to improve strategies for cardiomyocyte transplantation [50]. Intramyocardial injection of BMSCs with α-CD/MPEG–PCL–MPEG hydrogel can increase the survival and retention of transplanted cells and vessel density around the infarct zone when compared to BMSC implantation alone [63]. Injection of BMSCs with biomaterials significantly increased the LV ejection function and attenuated LV dilatation [63]. Hydrogel may also provide an ideal delivery system of growth factors for stem cell transplantation. Engineering vasculogenic endothelial cells and angiogenic effector cells with chitosan hydrogel containing VEGF-loaded microtubes significantly prolonged cell survival and effectively induced neovascularization and enhanced vascular repair [64]. Thus, a feasible strategy in cardiac muscle reconstruction would combine the bioengineering of therapeutic cells to facilitate vascularization while delivering them with a support system optimized for the damaged myocardium [60].

5 In Vitro Mimicry of Various Myocardial Stiffness and Preparation of Cell Culture Dishes

It has been well established that a standard culture system with rigid substrate does not effectively mimic the physical or mechanical microenvironment. Stem cells cultured on traditional plastic dishes concomitantly lose their self-renewal and regenerative potential rapidly, as shown by their limited contribution to muscle regeneration following transplantation [62, 65]. Excessive rigidification constrains cell-based cardiac repair by limiting favorable phenotype specifications [15]. Rowlands et al. [66] showed that PA gels containing singular ECM proteins of varying stiffnesses could affect MSC differentiation in a composition dependent manner. It is expected that the results obtained from flexible cell culture substrates will have significant implications for understanding physical effects of the in vivo microenvironment.

The following represents a method for preparing a series of hydrogel stiffness for testing the effects of microenvironment stiffness on survival, renewal, and differentiation of cultured stem cells in vitro. The variably compliant polyacrylamide hydrogels are prepared as previously described [67, 68].

5.1 Materials

5.1.1 Cleaning Cover Glass

1. NaOH, 1 N.
2. HCl, 1 N.
3. Distilled water.
4. 250 ml beaker.

5.1.2 Polyacrylamide Gel

1. No. 1.5 cover glass, 22 mm × 22 mm.
2. NaOH, 0.1 N.
3. PBS, 500 ml.
4. 3-aminopropyltrimethoxy saline.
5. Glutaraldehyde, 0.5 %, mix 357 μl of 70 % stock tightly sealed in zip bags in a closed container at 4 °C.
6. HEPES, 1 M, pH = 8.5.
7. HEPES, 50 mM, pH = 8.5.
8. Acrylamide (40 %).
9. Bis (2 %).
10. Ammonium persulfate (Bio-Rad) solution, 10 mg in 100 μl distilled water. Prepare immediately before use.
11. TEMED (Bio-Rad).
12. Sulfo-SANPAH (pierce), 0.5 mg/ml in 50 mM HEPES (pH = 8.5), need 200 μl per cover glass. Prepare immediately before use. Handle Sulfo-SANPAH in the dark. Weigh the appropriate amount, add 1 μl DMSO per mg of sulfo-SANPAH. While vortexing the DMSO and sulfo-SANPAH mixture, add 50 mM HEPES at room temperature to obtain the final concentration.

5.2 Gel Preparation

5.2.1 Cleaning Cover Glass

1. Add NaOH solution (1 N) to a beaker and seal the beaker with Parafilm.
2. Shake the beaker at 100 rpm for 4 h at RT.
3. Wash the cover glass with distilled water 3 times.
4. Add HCl solution (1 M) to the beaker and seal the beaker with Parafilm.
5. Shake the beaker at 100 rpm for 4 h.
6. Wash the cover glass with distilled water 3 times.
7. Add 100 % EtOH to the beaker, then shake for 2 min.
8. Put a piece of paper tissue in the fume hood; and put the cover glass separately on the paper tissue.
9. Wait until the cover glass dries.
10. Sterilize the cover glass through UV exposure for 15 min.
11. Store cover glass in a petri dish and seal the petri dish using Parafilm for future use.

5.2.2 Preparing Polyacrylamide Gel

1. Place the clean cover glass on plastic surface and add ~100 μl of 0.1 N NaOH to the cover glass, then smear the NaOH solution with cell scraper until the entire surface of the cover glass is covered. Wait until the NaOH solution dry.

2. Smear surface with ~100 μl 3-aminopropyltrimethoxy silane using a cell scraper, and then wait for 5 min at room temperature.
3. Remove the 3-aminopropyltrimethoxy silane by washing the cover glass with distilled water, and then wipe the cover glass with a Kimwipes to ensure that most of the silane is removed.
4. Collect the cover glass in a plastic container and rinse with distilled water. Place the plastic container on a shaker for 15 min. Remove the distilled water, replace with fresh distilled water, and shake for 15 min, repeat the washing four times.
5. Remove the distilled water and let the cover glass dry. Place the cover glass on aluminum foil or a metal surface, and then add 0.5 % glutaraldehyde to cover the cover glass. Wait for 30 min at room temperature.
6. Remove the glutaraldehyde and wash the cover glass with distilled water on a shaker. Make sure the treated surface of the cover glass is always facing up. The cover glass may be stored in desiccator for 2 weeks.
7. The stiffness of the PA gel depends upon the ratio of acrylamide to bis-acrylamide mix 5 ml of acrylamide solution in 15-ml Corning tubes according to Table 1.
8. Put the 15-ml Corning tubes in a vacuum chamber, and degas the solution for 60 min to remove the dissolved oxygen in the solution.
9. Add 30 μl freshly prepared ammonium persulfate solution, 20 μl TEMED to the Corning tubes, then seal the Corning tubes with Parafilm and swirl the mixture gently.
10. Transfer 15 μl the acrylamide solution to the activated surface of the cover glass, and then place an un-activated clean cover glass on top of the acrylamide solution droplet to make a cover glass–acrylamide solution–cover glass sandwich.

    (**Steps 9** and **10** should be finished within 2 min for all the cover glasses.)

Table 1
Acrylamide solution to make polyacrylamide gels of different stiffness

Young's Modulus (kPa)	40 % Acrylamide (μl)	2 % Bis (μl)	1 M HEPES (μl)	H_2O (μl)
75	1000	200	50	3750
30	1000	150	50	3800
10	1000	50	50	3900
24	625	200	50	4125
15	625	150	50	4175
7	625	63	50	4262

11. Wait until the acrylamide polymerizes. Use remaining acrylamide solution in the Corning tubes as a measure, until the acrylamide solution polymerizes to become gel.
12. Flood cover glass sandwich with 50 mM HEPES, and then remove the un-activated cover glass with fine tweezers.
13. Rinse the remaining activated cover glass with the formed polyacrylamide gel with 50 mM HEPES. The gel maybe stored at 4 °C for 2 weeks.
14. Remove as much liquid from the polyacrylamide surface as possible and add 100 μl sulfo-SANPAH solution.
15. Place under UV lamp for 5–8 min. The sulfo-SANPAH solution will become dark.
16. Repeat **steps 14** and **15**.
17. Rinse the polyacrylamide surface with 50 mM HEPES to remove excess sulfo-SANPAH solution. Do this quickly.
18. Add 100 μl fibronectin (20 μg/ml in PBS) to surface for 1 h at room temperature or overnight in 4 °C.
19. Before plating the cells, expose the surface in UV light for 15 min to sterilize the surface.
20. Rinse the surface with PBS.
21. The fibronectin-coated polyacrylamide surface is ready for cell culture.

6 Notes

1. Before being used for cell culture, the gels covered with PBS are exposed to ultraviolet light with wavelength of 275 nm for 15 min.
2. Thereafter PBS is replaced with complete culture medium and cell culture dishes are placed in incubator for 2 h to allow equilibrium.
3. Stem cells can be cultured in primary or passage culture.

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Chapter 24

Assessment of MiRNA Regulation of Endothelial Progenitor Cell Mediated Angiogenesis

Darukeshwara Joladarashi and Prasanna Krishnamurthy

Abstract

Organ outgrowth, embryonic development, wound healing, and many such processes require the process of angiogenesis, whereby new blood vessels are developed from the preexisting vessels. microRNAs (miRs) are 18–24 nucleotide-containing endogenous RNAs that, via a posttranscriptional mechanism, exert substantial gene regulatory effects. It was discovered by recent advances that, through direct targeting of certain critical secretory factors and transcription factors, miRs exert potent angiogenic control in a cell autonomous and non-cell autonomous manner. This chapter comprehensively summarizes step-by-step protocols for the (1) transfection of miRNA in EPCs (2) advantages and limitations of the principal tubule formation assays in use.

Key words MiRNA, Endothelial progenitor cells, Angiogenesis, Tubule formation, Matrigel

1 Introduction

Organ outgrowth, embryonic development, wound healing, and many such processes require the process of angiogenesis, whereby new blood vessels are developed from preexisting vessels [1–3]. Oxygen and nutrients are supplied to the tissues, waste products removed, and immune surveillance promoted by the newly evolved blood vessels that have a lining of endothelial cells [2–4]. All through fetal and embryonic development, angiogenesis holds a prime position; and in adults, this process remains in an inert state besides while skeletal growth, wound healing, pregnancy or during the menstrual cycle. The prominent molecular mechanisms regulating angiogenesis have begun to emerge. Pro- and anti-angiogenic signals like angiopoietins, integrins, chemokines, endogenous inhibitors, oxygen sensing agents, and junctional molecules balance the highly regulated event—Angiogenesis [5]. Proteases are released by the endothelial cells to degrade the basement membrane when the endothelial cell receptors are activated

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by basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), and epidermal growth factor (EGF); after which these cells proliferate and migrate at a rate of several millimeters per day forming sprouts [6, 7].

Many pathological conditions like psoriasis, cancer, arthritis, diabetic retinopathy, autoimmune disorders, infectious diseases, asthama, atherosclerosis, and arthritis are linked with angiogenesis [8–10]. For designing better therapeutics, awareness about the genes and pathways regulating the process of angiogenesis is crucial as the process is significant. To confirm the genes and pathways involved in the process of angiogenesis, tube formation assay is performed; it is a rapid and quantitative method. According to the principles of the assay, as first pronounced in 1988, the capability of the endothelial cells to divide and migrate briskly in response to angiogenic signals [11–13] is assessed. Also, the induced endothelial cells, when cultured on a matrix of basement membrane extract (BME), differentiate to form tube-like structures. The junctional complexes link the endothelial cells together that surround these tubes in a lumen. In this assay, tube formation is very rapid with many tubes forming within 2–6 h; this infact depends on the quantity and type of angiogenic stimuli.

To fine-tune the regulation of gene expression, miRNAs set up an essential regulatory network and hence maintaining cellular functions is necessary for an acceptable angiogenic response [14]. The extraordinary complexity and widespread number of miRNAs pledge the encounter of novel and unforeseen roles of miRNAs to control angiogenesis. Genomics efforts, such as massive parallel miRNA and mRNA expression profiling in angiogenic-associated diseases in combination with loss- or gain-of-functions screens in ECs, in combination with adequate target validation and large-scale proteomics are feasible approaches to help understand the complex miRNA-mediated gene regulatory networks in angiogenesis. In this chapter, we present a step-by-step protocol for the (1) EPCs isolation and culture, (2) transfection of miRNA in EPCs, and (3) procedure for tube formation assay. A brief outline of the procedure is presented in Fig. 1.

2 Materials

2.1 Method for Endothelial Progenitor Cells Isolation from Mouse Bone Marrow

Mice: 8–10-week-old C57BL/6J (stock number: 000664) male mice were purchased from Jackson Laboratory.

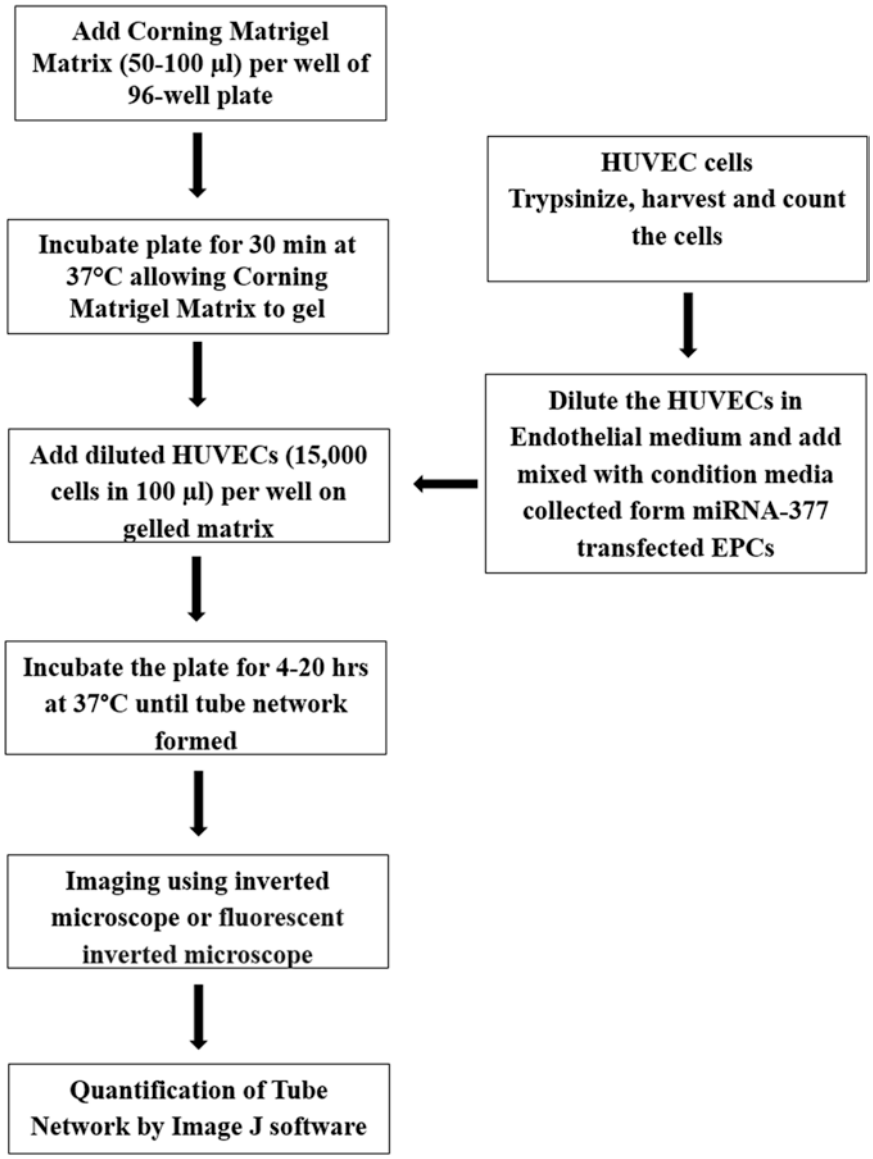

Fig. 1 Outline of endothelial cell tube formation assay

2.1.1 Reagents

DPBSE (*Ca^{++}; Mg^{++} free*)
5 mM EDTA.
Histopaque 1083.
Ammonium chloride (NH_4Cl).
Human fibronectin.
EBM-2 Bullet kit.
EBM-2 Basal Medium 500 mL.
EGM-2 Single Quot Kit suppl. and growth factors.
(Note: do NOT add hydrocortisone to the media).

2.1.2 Laboratory Equipment

6-well plates.

10 mL pipettes.

5 mL pipettes.

Screw cap sampling tubes (15 mL).

Screw cap sampling tubes (50 mL).

Cell strainer.

BD 20 mL syringe.

Pestle and mortar.

2.2 Procedure

1 Mice are sacrificed by CO_2 asphyxiation and cervical dislocation followed by spraying with 70 % ethanol. Blood is drawn as much as possible from mice. Femurs and tibias are harvested into cold PBS-EDTA on ice.

2 Note: Aseptic conditions must be maintained for further isolation procedure.

3 Bones will be gently squeezed in cold PBS-EDTA using pestle and mortar. Supernatant are collected in a 50 mL tube with 70 μm cell strainer using 18G syringe (10 mL). Repeat the above procedure, three times until the supernatant becomes clear and bone fragments are white (gradually apply more pressure to the bone fragments each time).

4 Total volume is made to 40 mL (add PBS-EDTA if needed). Carefully lay the cell suspension onto Histopaque 1083 (10 mL, brought to room temperature prior to laying cells) in a 50 mL conical tube. Centrifuge at 730 × *g*, 20 min, RT without brake.

5 Aspirate down to 15 mL, collect MNC layer (buffy coat) to new 15 mL tube with 18G syringe (approximately 3 mL). After adding 14 mL of PBS-EDTA, the conical tube is inverted and mixed. Centrifuge at 950 × *g*, 5 min, 4 °C with low brake. The pellet is washed by dissolving in 14 mL of PBS-EDTA. Centrifuge at 270 × *g*, 5 min, 4 °C with low brake.

6 The pellet is washed with 6 mL of NH_4Cl and 14 mL of PBS-EDTA, later it is centrifuged at 310 × *g* for 5 min at 4 °C, with low brake. The plates/dishes are coated with human fibronectin (5 μg/mL) for 1 h at 37 °C.

7 The pellet is dissolved in 4 mL EBM2 containing EGM2-MV Bullet kit medium (10 % FBS without hydrocortisone) and plated on 10 cm^2 dish without coating, later incubated at 37 °C for 30–40 min. The macrophage cells are depleted by allowing attachment to uncoated plate for 1 h. The supernatant contains EPC cells; this is counted and transferred appropriately to 6-well plate coated with 5 ug/mL human fibronectin (1 mL/per well).

8 Cells are cultured at 37 °C with 5 % CO_2 in a humidified atmosphere. After 4 days in culture, non-adherent cells are removed by washing with PBS, followed by addition of fresh media and the culture is maintained through day 7. EPCs, recognized as attaching spindle-shaped cells, are used for further analysis and treatment.

2.3 Transfection of miRNA-377 in Endothelial Progenitor Cells and Collection of Conditioned Media to Test for Its Angiogenic Potential

2.3.1 Reagents/ Chemicals

hsa-miR-377-5p mimic mirVana™ miRNA mimic (Ambion, Life Technologies).

mirVana™ miRNA mimic negative control (Ambion, Life Technologies).

hsa-miR-377-5p inhibitor mirVana™ miRNA inhibitor (Ambion, Life Technologies).

mirVana™ miRNA inhibitor negative control (Ambion, Life Technologies).

Lipofectamine® 2000 Transfection Reagent (Invitrogen).

2.4 Procedure

1. EPCs will be seeded in 6-well plates 24 h prior to transfection.
2. In a 0.5 mL Eppendorf tube (solution A) 6 μL of Lipofectamine and 0.5 mL optimum media are added.
3. In a separate 0.5 mL Eppendorf tube (solution B) 60 mM of miR-377 mimic or miR-377 inhibitor or respective negative control is added and incubated at RT for 10 min.
4. Then both solution A and B are mixed and further incubated for 30 min at RT.
5. After 30 min the transfection reagent mixture is added to prior coated EPC cells.
6. After 48 h, condition media is collected and its angiogenic potential is tested by vascular tube formation assay.
7. Use conditioned media immediately, or aliquot and store at −80 °C for several months.
8. Use non-conditioned native or low serum media as a negative control, and use non-conditioned complete growth media (10 % FBS, or appropriate concentration) as a positive control.

2.5 Vascular Tube Formation Assay

2.5.1 Materials

1. Reagents

   Corning Matrigel Matrix (Phenol red-free).

   Human Umbilical Vein Endothelial Cells (HUVECs; ATCC).

   Trypsin–EDTA solution, 1× (ATCC,).

   Phosphate-Buffered Saline, 1× (PBS).

   Endothelial Basal Medium-2 (EBM-2).

Dulbecco's Phosphate-Buffered Saline, 1× (DPBS).

Calcein AM (Trevigen).

Cultrex Cell Staining Solution (Trevigen,).

Methanol.

96-well cell culture plates.

15 mL conical centrifuge tubes-sterile.

Tissue culture flasks, 25 cm^2, filter cap, 50 mL.

Disposable sterile plastic pipettes.

2. Equipment

Cell culture incubator (humidified, 5 % CO_2).

Biological hood with laminar flow and UV light.

Pipette aid.

Sterile micropipette.

37 °C water bath

Centrifuge with a swing-bucket rotor, refrigerated.

Inverted phase microscope with 4× and 10× objectives (Zeiss).

Inverted phase microscope with fluorescence and 4× and 10× objectives (Olympus).

3 Procedure

3.1 Passaging of Human Umbilical Vein Endothelial Cells

TIMING 30 min (1 day before assay start)

HUVECs are split into in a 25-cm^2 flask containing nearly 5×10^5–1×10^6 cells using standard procedure. The HUVECs used for this assay should be used lesser than 12 passage and the cells should be passaged at least twice after removal from liquid nitrogen before being used in the assay.

3.2 Thaw the Corning Matrigel Matrix

TIMING 5 min (1 day before assay start)

Corning Matrigel Matrix is transferred to 4 °C from −20 °C or −80 °C before starting the assay. It is very important to maintain the cold temperature because Corning Matrigel Matrix solidifies very easily at room temperature. It is also important to keep the pipette tips in the cold conditions which are used to pipette the Corning Matrigel Matrix. It may be aliquoted and frozen at −20 °C or −80 °C; or stored at 4 °C for a few days.

3.3 Coating 96-well Cell Culture Plate with Corning Matrigel Matrix

TIMING 10–20 min

Before adding the Corning Matrigel Matrix label the well appropriately. Place a tube of fully thawed Corning Matrigel Matrix and labeled 96-well plate on ice in a laminar flow hood.

Load 50–80 μL of the Corning Matrigel Matrix per well of 96-well plate. While pipetting the Corning Matrigel Matrix care should be taken to avoid air bubbles. If any air bubbles get trapped in the well, the plate is centrifuged at 300×*g* for 10 min at 4 °C. Make sure that centrifuge is precooled to 4 °C before placing a plate with Corning Matrigel Matrix in it. The 96-well plate is incubated at 37 °C for 30 min in a cell culture incubator to allow the Corning Matrigel Matrix to gel. To maintain the even surface of the gel in 96-well plate it is important to avoid any shaking of the plate during gelling and transferring time.

3.4 Harvest 80 % Confluent HUVECs from 25-cm² Flask

TIMING 10 min

Before starting harvesting of HUVECs; warm the PBS, trypsin–EDTA, and EBM-2 in the 37 °C water bath. Remove and discard the media from 25-cm² flask with HUVECs and rinse cells with PBS. Add 1 mL of trypsin–EDTA to the flask, swirl briefly, and incubate at 37 °C for a few minutes to release the cells. Tap the side of the flask to be sure that the cells are detaching.

3.5 Quantitate and Collect Cells

TIMING 15 min

Once the cells are detached 4 mL of EBM-2 media is added and mixed gently. Pipette the solution up and down to make a single cell suspension. Transfer cell suspension in a sterile 15 mL conical tube. The cell number and cell viability are determined by mixing 5 μL of cell suspension with 5 μL of trypan blue and using a hemocytomoter. Typically, 1×10^6–1.5×10^6 cells is harvested from one 25-cm² flask at 80–90 % confluency. Cells pellet is collected by centrifuging at 200×*g* for 3 min. It is important to use HUVECs cells with a viability of more than 95 %.

3.6 Prepare Cells for Assay

TIMING 20 min

Pellet is resuspended in basal medium EBM-2 at a concentration of 1.5×10^5 cells per 1 mL. The cells are gently pipetted up and down a few times to obtain a single cell suspension.

3.7 Prepare Cells for Addition of Test Materials

TIMING 10–30 min

The 0.5 mL condition media collected from miRNA-377 mimic, inhibitor, and respective control transfected EPC is mixed with 1 mL of HUVECs (1.5×10^5 cells) and the cells are laid on top of gelled Corning Matrigel Matrix to begin the assay.

TIMING 15 min to add cells, 4–16 h incubation time

Gently add 100 μL (15,000 cells) per well of the single cell suspensions prepared in the above step to corresponding labeled wells of a 96-well plate on top of the gelled Corning Matrigel Matrix. HUVECs are mixed well before adding to the

96-well plate since cell density has an effect on tube formation. Do not touch the surface of the gel when adding the cells and add the cell suspension slowly so as not to disturb the gelled material. The 6-well plate is incubated at 37 °C, 5 % CO_2 in the cell culture incubator for a period of 4–16 h, or until the desired result is achieved. Examine the plate every hour for tube formation under an inverted microscope with 4× or 10× objectives. During the first hour of the tube assay, do not shake the plate or take it out of cell culture incubator. When observing the plate under a microscope, do not keep it longer than a minute or two outside the cell culture incubator.

3.8 Label and Image/ Quantitate Cells

TIMING 30–60 min

After confirming the HUVECs tube formation the cells can be viewed in the microscope to be photographed.

3.8.1 Microscopy

1. Photograph the tubular network in the wells using a digital camera attached to an inverted microscope with 4× or 10× objective.
2. Aspirate the medium from the wells, add 100 μL of warm DPBS and immediately photograph the wells. Replacing the medium with DPBS solution is necessary if the medium contains phenol red and the color reduces the quality of the pictures. Gently aspirate and load solutions so as not to disturb the network of endothelial cell tubes. Do not keep tubular network in DPBS for extended period of time, because it may detach from the matrix and start breaking apart.

HUVECs cell tube formation is also viewed by labelling with different stains depending upon the kind of microscope available.

3.8.2 Calcein AM

1. Prepare 6 μM of Calcein AM by adding 3 μL of 2 mM Calcein AM stock solution to 1 mL EBM-2 medium.
2. Without aspirating the medium, add 50 μL of 6 μM Calcein AM solution per well of the 96-well plate.
3. Incubate the plate at 37 °C and in 5 % CO_2 for 15–30 min.
4. Calcein AM-labeled cells are observed and photographed using a fluorescent inverted microscope with 485 nm excitation or 520 nm emission filter.

3.8.3 Fixation of Endothelial Cells to the Corning Matrigel Matrix Followed by Labeling with Cell Staining Solution

1. Aspirate the medium from the wells and rinse the wells three times with 100 μL of PBS per well. Gently aspirate and load solutions so as not to disturb the network of endothelial cell tubes.
2. Aspirate the last wash, add 100 μL of −20 °C cold methanol per well and incubate the plate for 30 s–1 min. Do not fix with methanol longer than 1 min, as it results in appearance of large

precipitates of basement membrane proteins in the matrix and interferes with the imaging process.

3. Aspirate methanol and immediately rinse the wells three times with distilled H_2O.
4. Aspirate last wash, add 100 μL of cell staining solution per well, and incubate the plate for 15–30 min at room temperature (21 °C).
5. Rinse the wells three times with distilled H_2O.
6. Endothelial cells are photographed and images are used for quantitation.

3.9 Quantification of Tube Network

1. Quantify the tube network in several different ways depending on computer programs with an ability to measure the following parameters: number of tubes; number of loops/meshes; number of branch sites/nodes; length of tubes.
2. Use representative computer program such as ImageJ with the Angiogenesis Analyzer plugin16 for quantification of tube networks.

4 Notes

1. The HUVECs used for this assay should be lesser than 12 passage and the cells should be passaged at least twice after removal from liquid nitrogen before being used in the assay. It is important to use HUVECs cells with a viability of more than 95 % [11].
2. It is very important to maintain the cold temperature because Corning Matrigel Matrix solidifies very easily at room temperature. It is also important to keep all the pipette tips in the cold conditions which are used to pipette the Corning Matrigel Matrix. It may be aliquoted and frozen at −20 °C or −80 °C; or stored at 4 °C for a few days [12].
3. If any air bubbles get trapped in the well, centrifuge the plate at 300×*g* for 10 min at 4 °C. Make sure that centrifuge is precooled to 4 °C before placing a plate with Corning Matrigel Matrix in it.
4. During the first hour of the tube assay, do not shake the plate or take it out of cell culture incubator. When observing the plate under a microscope, do not keep it longer than a minute or two outside the cell culture incubator.
5. Gently aspirate and load solutions so as not to disturb the network of endothelial cell tubes. Do not keep tubular network in DPBS for extended period of time, because it may detach from the matrix and start breaking apart [13].

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Chapter 25

Stem Cell Exosomes: Cell-FreeTherapy for Organ Repair

Mohsin Khan and Raj Kishore

Abstract

Cardiovascular disease is a significant cause of morbidity and mortality around the world. The damaged cardiac tissue is unable to repair itself following injury warranting the development of alternate therapies. Cell therapy has emerged recently as a viable treatment option resulting in significant improvement in cardiac function. Nevertheless, donated stem cells are hard to find in the heart after transplantation leading to the hypothesis that release of extracellular factors by stem cells is primarily responsible for the beneficial effect of cell therapy. Exosomes represent the bioactive component of stem cells and have been shown to recapitulate salutary effects of cell therapy on myocardial repair after injury. Here, we discuss the methodologies regarding the isolation and characterization of exosomes derived from stem cells.

Key words Regenerative medicine, Heart failure, Stem cells, Exosomes

1 Introduction

Advent of cell based therapies for tissue repair has ushered a new era for regenerative medicine. A overwhelming number of studies unequivocally correlate gains in tissue function directly with stem cell adoptive transfer. Nevertheless, there is some controversy surrounding the exact mode of action for the donated stem cell population. A large body of evidence implicates stem cell-derived small microvesicles including exosomes secreted under various physiological conditions that mediate the salutary effects of cell therapy [1, 2]. Therefore, it would be important to understand the sequential steps involved in the isolation, characterization, and functional applications of the stem cell-derived exosomes.

2 Materials

2.1 Materials for Electron Microscopy of Exosome Samples

1. Exosome pellet or suspension.
2. 2 or 4 % (w/v) paraformaldehyde (PFA).
3. Phosphate-buffered saline (PBS).

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4. 1 % glutaraldehyde.
5. Formvar-carbon coated EM grids.
6. Parafilm.
7. Forceps (Dumont No. 5), clean.
8. Glass dish.
9. Grid storage boxes.
10. Transmission electron microscope (TEM).

2.2 Materials for Ultracentrifugation Based Exosome Isolation

1. Stem cell conditioned medium.
2. Phosphate-buffered saline (PBS).
3. Refrigerated centrifuge.
4. 50-ml polypropylene centrifuge tubes.
5. Ultracentrifuge and fixed-angle or swinging-bucket rotor.
6. Polyallomer tubes or polycarbonate bottles, appropriate for the ultracentrifuge rotor.
7. Micropipettor (e.g., Pipetman).
8. Tabletop ultracentrifuge (e.g., Beckman TL-100).
9. –80 °C freezer.

2.3 Materials for Western Blot Analysis of Exosomes

1. Exosomes pellet or suspension.
2. Phosphate-buffered saline (PBS).
3. 4× SDS sample buffer, reducing or non-reducing (i.e., with or without DTT or 2-mercaptoethanol).
4. Additional reagents and equipment for protein quantification.
5. SDS-PAGE and western blots.

3 Exosome Isolation

3.1 Stem Cell Culture

1. Different stem cell types are cultured in appropriate cell culture medium to desired confluency.
2. Growth medium is replaced by "conditioned medium" containing necessary growth supplements but importantly the fetal bovine serum (FBS) is replaced by exosome-free or knockout FBS. Cells are allowed to grow for 24–48 h followed by media collection. Medium can be stored at 4 °C for short term but should be stored at −80°°C for long-term storage.

3.2 Ultracentrifugation

One of the most reliable methods of exosome isolation is based on ultracentrifugation [3, 4]. The resultant exosome preparations are largely free from contaminating soluble proteins, growth factors, or cellular debris.

1. Tilt and gently swirl the plate to resuspend exosomes. Pipet up and down to further resuspend exosomes, and then wash the plate with culture media.
2. For attached cells, tilt and swirl the plate and remove the media.
3. Spin cells down at 500 × *g* for 5 min; take the supernatant.
   Optional: to remove dead cells, spin at 3000 × *g* for 5 min. Take the supernatant.
4. Fill the centrifuge tubes with about 22 mL of media each and be sure to balance the tubes. Fill any open slots in the rotor with blank tubes (water).
5. Draw a small dot on the tube where the plastic seams meet. Align this dot to the outmost edge of the rotor for all spins.
6. Spin the media down at 20,000 × *g* (14,000 rpm) for 20–30 min at 4°C in the Ti70 rotor.
7. Near the centrifuge, carefully remove the supernatant from the tubes and transfer to 50 mL conical tubes.
8. In clean centrifuge tubes, add 3–4 mL of 30 % sucrose. Very carefully (set pipette to slowest setting and hold the tip close to the top of the sucrose) layer the media on top of the sucrose.
9. Balance all the tubes and carefully load them into the rotor and centrifuge.
10. Centrifuge at 120,000 × *g* (35,000 rpm) for 55–70 min.
11. Take out as much supernatant from above the sucrose layer as possible. Transfer the supernatant to 50 mL conical tubes.
12. To the sucrose, add ~20 mL of PBS to fill the tubes. Balance and Centrifuge at 120,000 × *g* (35,000 rpm) for 55–70 min.
13. Remove the supernatant. Tip the tube upside-down for 2–3 min to allow excess liquid to drain out. Blot any excess liquid with a Kimwipes.
14. Resuspend the pellet in PBS (should be near the dot on the tube), washing the area twice.

3.3 Kit Based Exosome Isolation

In contrast to the ultracentrifugation based procedure, exosomes can be isolated with ExoQuick-TC (Systems Biosciences) [5] according to the manufacturer's protocol. Some of the steps are described below:

1. Collect the conditioned medium and centrifuge at 3000 × *g* for 15 min to remove cells and cell debris.
2. Transfer supernatant to a sterile tube and add the appropriate volume of ExoQuick-TC Exosome Precipitation Solution (2 mL ExoQuick-TC for every 10 mL of medium) to the conditioned medium. Mix well by inverting or flicking the tube. Keep the tubes overnight at 4 °C.

3. Centrifuge the Exoquick-TC/medium mix at 1500 × *g* for 30 min. Centrifugation may be performed at either room temperature or 4 °C with similar results. After centrifugation, the exosomes may appear as a beige or white pellet at the bottom of the tube.
4. Aspirate supernatant. Spin down residual ExoQuick-TC solution by centrifugation at 1500 × *g* for 5 min. Remove all traces of fluid by aspiration, taking great care not to disturb the precipitated exosomes in pellet.
5. Resuspend exosome pellet in 100–500 μL of buffer. Please see the next Subheading 4 of this protocol to determine the appropriate buffer for protein or RNA analysis.

4 Exosome Characterization

Isolated exosome need to be characterized in order to determine their whether there are any impurities in the exosome preparation. Characterization methods are based on three things: careful analysis typical exosome cell surface markers [6, 7], electron microscopy [4], and exosome size analysis [1].

4.1 Western Blot

1. Quantify exosome preparation by using the commonly used protein quantification methods such as Pierce BCA protein estimation kit (Thermo Scientific).
2. Analyze the exosome protein by an immunoblot and determine the expression of common exosome markers such as flotillin-1, CD63, and CD81.

4.2 Electron Microscopy

1. Exosomes are fixed with 2 % paraformaldehyde, loaded on 300-mesh formvar/carbon coated electron microscopy grids (Electron Microscopy Sciences, USA).
2. Post-fixed in 1 % glutaraldehyde, and then contrasted and embedded as described previously [4].
3. Transmission electron microscopy images are obtained with an FEI (Hillsboro, OR, USA) Tecnai Spirit G2 transmission electron microscope operating at 120 kV.

4.3 Dynamic Light Scattering

1. Exosomes are suspended in phosphate-buffered saline (PBS) containing 2 mM ethylenediaminetetraacetic acid (EDTA).
2. Dynamic light-scattering measurements are performed with a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) as described previously [3].
3. Intensity, volume, and distribution data for each sample are collected on a continuous basis for 4 min in sets of three.

4. At least three different measurements from three different samples are performed for each exosome population.

5 Whole Exosome Labeling

In order to determine whether target cells effectively take up exosomes, they can be labeled by fluorescent dye such as PKH-26 (Sigma Aldrich). Additionally, exosome RNAs and proteins can be separately labeled using Exo-Glow (Systems Biosciences).

5.1 PKH-26 Exosome Labeling

1. Prepare a 2× exosome suspension by adding 1 mL Diluent C (PKH-26 kit, Sigma Aldrich) and gently mix.
2. Immediately prior to staining, prepare a 2× dye solution in diluent C by adding 4 μL of the PKH-26 dye solution to 1 mL of the diluent C and mix well.
3. Add the staining solution directly to the exosome suspension and mix well.
4. Incubate the exosome suspension in the staining solution for 5 min and room temperature.
5. Stop the staining by adding equal volumes of 1 % BSA and incubate for 1 min for the excess dye to bind to the BSA.
6. The exosome are now labeled and ready to use. However, the exosome preparation will be diluted now and can be used directly for further assays. In contrast, exosome suspension can be centrifuged at 120,000 × *g* for 60–70 min to pellet the exosomes followed by their resuspension in appropriate medium according to the required concentration.

5.2 Labeling of Exosome Contents

Exosome RNA and protein can be labelled by using Exo-Glow kit (System biosciences) according to the manufacturer's protocol. In brief, some of the steps are outlined below:

1. Add 50 μL 10× Exo-Red or Exo-Green to 500 μL volume of exosome suspended in PBS.3.2.2. Mix it well by flicking/inversion. Do not vortex.
2. Incubate the exosome solution in 37 °C for 10 min (rotation not necessary).
3. To stop labeling reaction, add 100 μL of the ExoQuick-TC reagent to the labeled exosome sample suspension and mix by inverting six times.
4. Place the labeled exosome sample on ice (or at 4 °C) for 30 min. Centrifuge the sample for 3 min at 20,000 × *g* (14,000 rpm) in a microfuge.
5. Remove the supernatant with excess label and resuspend the labeled exosome pellet in 500 μL 1× PBS.

6 Applications for Cardiac Regeneration

Stem cell-derived exosomes possess immense regenerative potential for the repair of damaged myocardium. In vitro application: Repair potential of stem cell exosomes can be assessed in vitro by carrying out a number of simple assays. Analysis of exosome content can be done to show enrichment of specific proteins, mRNAs, and miRNAs within exosomes [8, 9]. Additionally, stem cell exosomes can be applied to human umbilical vein endothelial cells (HUVECs) to determine the exosome ability to influence tube formation and angiogenesis. Similarly, treating a particular cell line with stem cell exosomes parallel with H_2O_2 challenge can help in conducting cell survival studies. In vivo application: Studies with different stem cells have demonstrated that the transplanted cells release exosomes at the site of injury leading to augmentation of cardiac function. There are a number of different delivery routes with studies utilizing both direct intramyocardial [1, 2] and intracoronary [10, 11] exosome delivery. There some dose variations as well and the summary of the literature shows a dose ranging from 50 to 100 μg/animal has been administered to animals with myocardial infarction at the time of LAD ligation [1, 11]. Analysis of the stem cell exosome content has revealed accumulation of many mRNAs, proteins and miRNAs specific to the parent cell of origin. Once in the tissue, stem cell exosomes are able to release these contents resulting in activation of resident cardiac stem cells [1, 12], cardiomyocyte survival and proliferation [13, 14], and neovascularization [15, 16]. Consequently, there is a significant augmentation of cardiac function in the animals receiving exosomes, thus providing evidence for future studies utilizing exosomes.

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INDEX

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