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Chrissa Kioussi *Editor*

Stem Cells and Tissue Repair

Methods and Protocols

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Stem Cells and Tissue Repair

Methods and Protocols

Edited by

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 **Humana Press**

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Preface

The goal of regenerative medicine is to rejuvenate an aging, or a sick, body by one of the three approaches. First, its latent regenerative capacity can be stimulated in a targeted way. Second, replacement organs can be grown *de novo* and surgically implanted. Third, tissue can be surgically implanted and coaxed to integrate and restore problem areas. The first approach will be aided by an accurate mechanistic understanding of the unique molecular process that takes place in each adult stem cell/niche microenvironment as it repairs and regenerates its corresponding tissue/organ during the normal life-span. The second approach essentially mimics embryonic development in a technologically advanced “incubator” and will be aided by an accurate mechanistic understanding of the unique cellular and molecular process that takes place as each organ is formed in embryos. Understanding the particulars of adult stem cell microenvironments and embryonic development is important because it will allow interventions and replacements to be designed and tested on a rational basis. Fundamental to developing this understanding is that the traditional language used to identify cell types (morphological, functional, and marker) is gradually translated into a new “language” that identifies/defines each “cell type” by the network kernel that stabilizes its gene regulatory state. Vigorous basic research must continue to develop suitable representations of the states and their transitions in computational models that humans can collectively refine across generations. The third approach is decidedly more empirical, and like much of medicine, it addresses our desire to help those in need quickly. It will no doubt benefit from more basic research, but it will also inform basic research by providing an immediate proving ground in a medical forum. A balanced approach between developing a basic understanding of process, developing suitable technology, and taking swift decisive action will likely benefit us most. In this sense, chapters have been commissioned on the methods used in all three approaches.

The first approach taps into the latent regenerative capacity of particular tissues, such as muscle (Chapters 5 and 6), skin (Chapter 12), fat (Chapter 13), or bone marrow (Chapter 5), and organs such as teeth (Chapters 8 and 9), testis (Chapter 15), and hair follicles (Chapter 16). The second approach induces and grows pluripotent stem cells (Chapters 1 and 11); drives their differentiation along certain pathways such as germ layers (Chapter 1), neural crest (Chapter 7), liver (Chapter 10), teeth (Chapters 8 and 9), or retina (Chapter 14); and cultures organs such as pancreas (Chapter 17) and heart (Chapter 18) from them in specific ways. The third approach involves various engraftment techniques for neural tissue (Chapters 2–4). The articles themselves will provide state-of-the-art method descriptions, and the references therein will provide a suitable starting point for exploring the vast literature that has already been developed for regenerative medicine.

Corvallis, OR, USA

Chrissa Kioussi

Contents

<i>Preface</i>	<i>v</i>
<i>Contributors</i>	<i>ix</i>
1 Culturing and Differentiating Mouse Embryonic Stem Cells	1
<i>Axel P. Gross and Chrissa Kioussi</i>	
2 Neural Stem Cell Transplantation in an Animal Model of Traumatic Brain Injury	9
<i>Dimitra Thomaidou</i>	
3 Experimental Cell Transplantation for Traumatic Spinal Cord Injury Regeneration: Intramedullar or Intrathecal Administration	23
<i>Ana Alastrue-Agudo, Slaven Erceg, Marta Cases-Villar, Viviana Bisbal-Velasco, Richard J. Griffeth, Francisco Javier Rodriguez-Jiménez, and Victoria Moreno-Manzano</i>	
4 Generation of Murine Xenograft Models of Brain Tumors from Primary Human Tissue for In Vivo Analysis of the Brain Tumor-Initiating Cell	37
<i>Maleeha Qazi, Aneet Mann, Randy van Ommeren, Chitra Venugopal, Nicole McFarlane, Parvez Vora, and Sheila K. Singh</i>	
5 Growth of Bone Marrow and Skeletal Muscle Side Population Stem Cells in Suspension Culture	51
<i>Christina A. Pacak and Douglas B. Cowan</i>	
6 Isolation, Culture and Immunostaining of Skeletal Muscle Fibres to Study Myogenic Progression in Satellite Cells	63
<i>Louise A. Moyle and Peter S. Zammit</i>	
7 Human Neural Crest Stem Cells Derived from Human Pluripotent Stem Cells	79
<i>Qinyue Liu, Andrzej Swistowski, and Xianmin Zeng</i>	
8 Dental Pulp Stem Cell (DPSC) Isolation, Characterization, and Differentiation	91
<i>Federico Ferro, Renza Spelat, and Chelsea S. Baheney</i>	
9 Dental Pulp Stem Cells Isolation and Osteogenic Differentiation: A Good Promise for Tissue Engineering	117
<i>Adriana Di Benedetto, Claudia Carbone, and Giorgio Mori</i>	
10 Efficient Hepatic Differentiation of Human Induced Pluripotent Stem Cells in a Three-Dimensional Microscale Culture	131
<i>Ran-Ran Zhang, Takanori Takebe, Leina Miyazaki, Maho Takayama, Hiroyuki Koike, Masaki Kimura, Masahiro Enomura, Yun-Wen Zheng, Keisuke Sekine, and Hideki Taniguchi</i>	
11 The Generation and Maintenance of Rat Induced Pluripotent Stem Cells	143
<i>Tomoyuki Yamaguchi, Sanae Hamanaka, and Hiromitsu Nakauchi</i>	

12	Protocol for Cutaneous Wound Healing Assay in a Murine Model.	151
	<i>Gitali Ganguli-Indra</i>	
13	Adipose-Derived Stem Cells: Methods for Isolation and Applications for Clinical Use.	161
	<i>Brian Mailey, Ava Hosseini, Jennifer Baker, Adam Young, Zeni Alfonso, Kevin Hicok, Anne M. Wallace, and Steven R. Cohen</i>	
14	In Vitro Detection of Residual Undifferentiated Cells in Retinal Pigment Epithelial Cells Derived from Human Induced Pluripotent Stem Cells.	183
	<i>Takuya Kuroda, Satoshi Yasuda, and Yoji Sato</i>	
15	Whole-Mount Immunohistochemistry to Study Spermatogonial Stem Cells and Spermatogenic Lineage Development in Mice, Monkeys, and Humans.	193
	<i>Kathrin Gassei, Hanna Valli, and Kyle E. Orwig</i>	
16	Differentiating the Stem Cell Pool of Human Hair Follicle Outer Root Sheath into Functional Melanocytes	203
	<i>Marie Schneider, Christina Dieckmann, Katrin Rabe, Jan-Christoph Simon, and Vuk Savkovic</i>	
17	Pancreas Development Ex Vivo: Culturing Embryonic Pancreas Explants on Permeable Culture Inserts, with Fibronectin-Coated Glass Microwells, or Embedded in Three-Dimensional Matrigel™.	229
	<i>Hung Ping Shih and Maike Sander</i>	
18	Ultra-rapid Manufacturing of Engineered Epicardial Substitute to Regenerate Cardiac Tissue Following Acute Ischemic Injury	239
	<i>Vahid Serpooshan and Pilar Ruiz-Lozano</i>	
	<i>Index.</i>	249

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Chapter 1

Culturing and Differentiating Mouse Embryonic Stem Cells

Axel P. Gross and Chrissa Kioussi

Abstract

Pluripotent embryonic stem (ES) cells have been used extensively for over 20 years for creating mouse mutants as models for developmental biology and human diseases. The genetic manipulations of the ES cells have revolutionized our understanding of organ development and abilities to genetically manipulate the mouse embryo. Understanding the ES cell differentiation has provided new insights essential for establishing cell-based therapy and tissue regeneration.

Key words Mouse embryonic stem cells, Differentiation, Mouse embryonic fibroblasts

1 Introduction

Embryonic stem (ES) cells are karyotypically normal pluripotent cells derived from the inner cell mass of blastocyst-stage embryos with the ability to differentiate in any adult somatic lineages in vivo and in vitro [1, 2].

They can self-renew symmetrically and maintain their karyotype and function indefinitely [3]. The pluripotent nature of mouse ES cells was demonstrated by their ability to contribute to germ-line and adult tissues after injection into host blastocysts [4]. ES cells have also the remarkable ability to differentiate into specific lineages when in culture [5] and thus make them a unique system for establishing in vitro models for early mammalian development, cell replacement therapy, and tissue regeneration, with applications in drug discovery.

ES cells can differentiate to all three germ layers, mesoderm, ectoderm and endoderm, under appropriate conditions [5]. Three differentiation strategies are typically used to differentiate ES cells: (1) ES can aggregate and form three-dimensional colonies, the embryoid bodies (EBs) [6], (2) when cultured directly on stromal cells [7] and (3) when cultured as a layer on extracellular matrix proteins [8].

ES cells have initially been established and maintained in their undifferentiated state in co-culture with mouse embryonic fibroblasts (MEFs) [1, 2]. The leukemia inhibitory factor (LIF), a feeder-derived molecule, promotes the proliferating and non-differentiating state of ES [9] and can replace the MEF feeder layer in the presence of fetal bovine serum (FBS) [9, 10]. Addition of the bone morphogenetic factor (BMP) 4 in the presence of LIF is capable to replace the serum requirement. Several transcription factors, including Oct3/4 [11, 12], Sox2 [13], and Nanog [14, 15], maintain the pluripotency of early embryos and ES cells. Several genes that are frequently present in high levels in tumors are also contributing to the long-term maintenance and proliferation of ES cells.

In this chapter we provide protocols for maintenance and expansion of ES cells currently used for transgenic and gene knockout for the analysis of gene function. The in vitro differentiation protocols of mouse ES cells are used to further understand the molecular mechanisms involved during organ development.

2 Materials

2.1 Tissue Culture Equipment

1. Incubator, humidified 37 °C, 5 % CO₂, 95 % air.
2. Inverted microscope.
3. Microcentrifuge.
4. Microcentrifuge tubes, 1.5 ml.
5. Electroporator.
6. Test tubes, 15 ml.
7. Tissue culture dishes 15 cm, 10 cm, 6 cm, 3.5 cm, 4-well, 24-well, 96-well.
8. Glass cover slips.
9. Bacteria grade plastic dishes, 10 cm, 6 cm, 3.5 cm.
10. Cuvette, 0.4 cm.
11. Automated pipettes, multichannel pipettes, various sizes.

2.2 Tissue Culture Reagents and Supplies

1. Mouse embryos day 13.5.
2. MEF medium: DMEM high-glucose medium supplemented with 15 % fetal bovine serum (FBS), nucleosides, 2 mM glutamine, 0.1 g/ml penicillin/streptomycin, 0.002 % β -mercaptoethanol.
3. FBS, fetal bovine serum (*see Note 1*).
4. Leukemia inhibitory factor (LIF), 500–1,000 units/ml.
5. ES medium: MEF medium with LIF.
6. Trypsin 0.25 % (w/v)/EDTA 0.04 % (w/v).

7. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.76 mM KH₂PO₄ in ddH₂O, pH 7.4 (with HCl).
8. Mitomycin C (10 µg/ml) (*see Note 2*).
9. Gelatin (0.1 % in ddH₂O, autoclaved).
10. EmbryoMax[®] Electroporation Buffer.
11. Neomycin (G418; 250 µg/ml).
12. Hygromycin B (50 µg/ml).
13. All-trans retinoid acid 10⁻² M dissolved in dimethyl sulfoxide. Split into 100 µl aliquots and store in the dark at -80 °C.
14. Laminin (20 µg/ml) (*see Note 3*).
15. Poly-L-ornithine hydrochloride (1.5 µg/ml) (*see Note 4*).
16. 3,3',5-triiodo-L-thyronine (T3) (20 ng/ml) (*see Note 5*).
17. Human recombinant insulin (500 µl/ml).

3 Methods

3.1 Preparation of Mouse Embryonic Fibroblasts

1. Aseptically dissect mouse embryos at days 13.5–15.5: usually two pregnant mice (about 12 embryos per litter) (*see Note 6*).
2. Remove yolk sac, head, and organs. Isolate limbs with some parts of the body, wash with PBS, and place into a 15 ml tube containing 3 ml 0.25 % trypsin/EDTA. Incubate at 37 °C, 5 % CO₂, 95 % air for 20 min. Invert gently every 5 min (*see Note 7*).
3. Stop the reaction by adding 3 ml FBS. Centrifuge cells at 300×g for 5 min.
4. Remove supernatant, and resuspend pellet in 1 ml of MEF medium. Pipette up and down to mix (*see Note 8*).
5. Count cells and dilute them to a final density of 2 × 10⁵/ml (*see Note 9*).
6. Incubate cells O/N at 37 °C, 5 % CO₂, 95 % air.
7. The next day remove medium from plates, and wash twice with PBS.
8. Dissociate cells by adding 1 ml of trypsin/EDTA and incubate at 37 °C, 5 % CO₂, 95 % air for 4 min.
9. Stop trypsin activity by adding 2 ml of medium and transfer to a 15 ml test tube.
10. Centrifuge at 300×g for 5 min.
11. Remove supernatant and resuspend pellet in 7 ml of medium by gentle pipetting.
12. Place 1 ml in 6 cm and in 3.5 cm gelatinized plates (*see Note 10*).

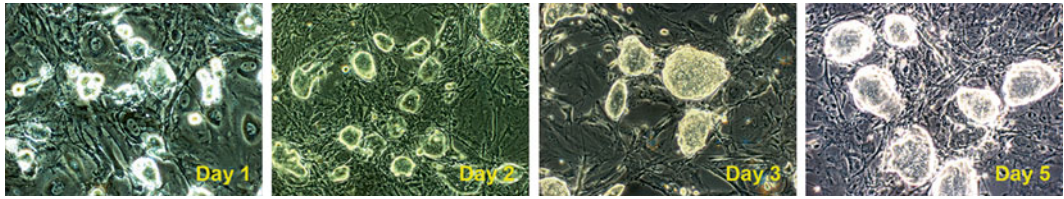


Fig. 1 Undifferentiated mouse ES cells growing in an MEF feeder layer in the presence of serum and LIF. Note the well-rounded cell colonies with defined borders

3.2 Grow and Passage ES Cells

1. Thaw a vial of ES cells (*see Note 11*) by quickly warming it in a 37 °C water bath.
2. Aseptically transfer cells into a 15 ml tube, add 2 ml of media, and centrifuge for 5 min at 300 × *g*.
3. Remove supernatant. Resuspend in 4 ml of media, and plate on a 3.5 cm dish with MEFs.
4. Change medium the next day. When the culture reaches 70 % confluence, aspirate the medium, and rinse dish with PBS.
5. Add 0.3 ml trypsin/EDTA and incubate at 37 °C, 5 % CO₂, 95 % air for 5 min. Check in the microscope for detached clumps.
6. Stop reaction by adding 2 ml of ES medium. Transfer to a 15 ml tube, and centrifuge for 5 min at 300 × *g*. Aspirate supernatant and resuspend in fresh ES medium.
7. Count cells and plate them on MEF feeder dishes, 10⁶ cells/6 cm dish or 2 × 10⁶ cells/10 mm dish.
8. Change media daily, and keep track of the passaging number.
9. Passage ES cells every 2–3 days for maintenance of freezing. Check ES cells in microscope daily before and after changing medium (Fig. 1).

3.3 Freeze ES Cells

1. Prepare fresh freezing medium, by adding 10 % FCS and 10 % DMSO in ES medium (*see Note 12*).
2. Remove medium from dish, wash cells with PBS, add trypsinize cells as indicated.
3. Resuspend ES cells in the appropriate volume of a cold freezing medium to reach a concentration of 5 × 10⁶ cells/ml/vial.
4. Place vials in a cryo-container at –80 °C freezer for 24 h. Transfer vials to liquid nitrogen container for long-term storage.

3.4 Electroporate ES Cells

1. Three days before electroporation is to be performed, prepare 8 × 10 cm plates with MEF cells (as described in Subheading 3.1).
2. The morning that electroporation is to be performed feed the ES cells fresh medium.

3. After 2–3 h harvest ES cells (as described in Subheading 3.1), and determine the cell count. 1×10^7 ES cells is the minimum number of cells required for electroporation. Freeze any excess of ES cells (as described in Subheading 3.2).
4. Centrifuge ES cells required for electroporation at $300 \times g$ for 10 min, aspirate, and discard medium.
5. Resuspend ES cells in 600 μ l of electroporation buffer.
6. Add 25–40 μ g of knockout linearized DNA vector (purified) dissolved in 30 μ l of electroporation buffer to the ES cells. Mix well and leave for 5 min at room temperature.
7. Place the ES cells in a 0.4 cm electroporation cuvette. Electroporate at 500 μ FD, 0.24 kV. The time constant produced should be around 7 ms. Place cuvette on ice for 10 min.
8. Transfer electroporated ES cells to 80 ml of ES medium.
9. Mix gently. Plate ES cells, 10 ml per feeder plate.
10. Incubate for 24 h at 37 °C, 5 % CO₂, 95 % air prior to antibiotic selection.

3.5 Select and Pick ES Cell Colonies

1. Transformant ES cells are usually selected for resistance to neomycin (G418) or hygromycin B.
2. After 48 h, cell death should be apparent. Change media daily. ES cell-resistant colonies should reach 800 cells in approximately 7 days following electroporation.
3. The day prior to picking ES cell colonies, prepare appropriate number of 24-well plates with and without MEFs.
4. Colonies selected for picking should be spaced well enough apart to ensure no contamination from surrounding colonies with defined borders. Set pipette to 15 μ l, scrape the colony with the pipette tip to dislodge the colony, aspirate, and transfer the colony to an empty well in a 96-well plate (*see Note 13*).
5. Use a new tip for each colony. Usually 20–30 ES colonies can be picked from one 10 cm plate.
6. Add 5 μ l of trypsin to each well and incubate at 37 °C, 5 % CO₂, 95 % air for 2 min. Replace MEF media in the 24-well plates with 500 μ l of ES cell medium.
7. Disperse ES cell colony in the 96-well plate by using a multi-channel pipette to break up each colony. Transfer the suspension to the 24-well plate containing 500 μ l of ES cell medium.
8. Remove 250 μ l of ES cells, transfer to a new 24-well plate without MEFs, and add 250 μ l ES medium in both plates with and without MEFs. The plate without MEFs will be used for genotyping while the one with MEFs will be stored for further use and ES expansion.

9. Change media daily with medium supplemented with neomycin or hygromycin (50 µg/ml).
10. New colonies should be evident within a few days. If colonies are too close disperse them by breaking the colonies using a pipette tip and spread the cells. Each well should be covered with colonies in 7–10 days.

3.6 Differentiate ES Cells into Embryoid Bodies (EB)

1. Resuspend ES cells in MEF media (without LIF).
2. Plate ES cells onto bacterial grade dishes, 5×10^5 cells per 10 cm dish (*see Note 14*) (day 0).
3. Two days later change media (day 2). Tilt plate and aspirate very carefully the media. Cells are detached and easy to be aspirated.
4. Change media every day as in day 2. As ES cells differentiate they form bigger clumps and that settle to the bottom.
5. Keep ES cells up to 2 weeks. At day 15, EBs develop cavities that are filled with fluid and these structures are now called cystic EBs.

3.7 Neuronal Induced ES Cells

1. For induction of neurogenesis, EBs were collected (as described in Subheading 3.6) and incubated in 10 ml MEF medium in the presence of 2 µl of 500 µl RA.
2. Change media daily for a total of 4 days.
3. Collect EBs in a 15 ml tube, let them settle to the bottom of the tube, and aspirate media carefully.
4. Plate 10–20 EBs onto a laminin/poly-L-ornithine-coated glass inside a chamber of a 4-well plate.
5. Change MEF medium in the presence of 2 µl of 500 µl RA every 2 days for a total of 8 days. Neuronal like cells will appear around day 4 (*see Note 15*).

3.8 Adipocyte-Induced ES Cells

1. For induction of adipogenesis, EBs were collected (as described in Subheading 3.6) and incubated overnight in 10 ml MEF medium in the presence of 2 µl of 500 µl RA.
2. Change media daily for a total of 3 days.
3. Collect EBs in a 15 ml tube, let them settle to the bottom of the tube, and aspirate media carefully.
4. Plate 10–20 EBs onto a gelatin-coated glass inside a chamber of a 4-well plate.
5. Change MEF medium in the presence of 20 ng/ml T3 and 500 µl/ml insulin, every 2 days for a total of 8 days. Adipocyte-like cells will appear around day 4 (*see Note 16*).

4 Notes

1. Serum needs to be tested for its effect in ES differentiation.
2. If MEFs will be used as feeders for ES using media with antibiotic selection such as G418, then mice that carry G418-resistant gene need to be used.
3. Recombinant laminin is stored at -80°C . Thaw vial before use and dilute in PBS. Used freshly diluted laminin all times. Apply 300 μl laminin on each glass cover slip, and incubate at 37°C for at 2 h.
4. Prepare 1.5 mg/ml (100 $\times$) aliquots of poly-L-ornithine hydrochloride by diluting in H_2O . Store at -20°C .
5. To prepare 20 $\mu\text{g}/\text{ml}$ stock solution add 1 ml 1 N NaOH per 1 mg 3,3',5-triiodo-L-thyronine; gently swirl to dissolve and add 49 ml DMEM medium. Stock solutions are stored at -20°C . Working solutions are stable for 30 days at 4°C .
6. Store as solution 1 mg/ml in PBS for 1 week at 4°C protected from light. Wear gloves for handling.
7. The medium should be cloudy; if not incubate for another 5–10 min.
8. Supernatant will be gooey and difficult to remove with a pipette. A cut of the pipette tip is suggested for better aspiration.
9. One 15 cm confluent plate can generate five 10 cm or ten 6 cm or twenty 3.5 cm plates.
10. If MEFs are used for feeders then they need to be plated onto gelatinized dishes. Gelatinized dishes can be prepared the day before by adding 1 ml, 2 ml, and 4 ml 0.1 % gelatin in 3.5 cm, 6 cm, and 10 cm dishes, respectively, for at least 3 h. Aspirate gelatin and let dishes dry inside the biosafety cabinet. Dry gelatinized dishes can be stored up to 2 weeks.
11. Different strains of mouse ES cells are commercially available.
12. The final concentration of FBS of the freezing medium should be 30 %.
13. Before start picking ES cell colonies, ensure that you are wearing gloves and facemask. All surfaces including the microscope should be wiped with ethanol prior to use.
14. Cell density influences ES cell differentiation. Several cell concentrations can be used as pilot studies.
15. Cells can be fixed and immunolabelled with anti- β III-tubulin to observe the neuronal like state.
16. Cells can be fixed and stained with Oil Red to confirm the lipid-containing adipocytes.

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Chapter 2

Neural Stem Cell Transplantation in an Animal Model of Traumatic Brain Injury

Dimitra Thomaidou

Abstract

The central nervous system (CNS) can be damaged by a wide range of conditions resulting in loss of specific populations of neurons and/or glial cells and in the development of defined psychiatric or neurological symptoms of varying severity. As the CNS has limited inherent capacity to regenerate lost tissue and self-repair, the development of therapeutic strategies for the treatment of CNS insults remains a serious scientific challenge with potential important clinical applications. In this context, strategies involving transplantation of specific cell populations, such as stem cells and neural stem cells (NSCs), to replace damaged cells offers an opportunity for the development of cell-based therapies. Along these lines, in this review we describe a protocol which involves transplantation of NPCs, genetically engineered to overexpress the neurogenic molecule Cend1 and have thus the potency to differentiate with higher frequency towards the neuronal lineage in a rodent model of stab wound cortical injury.

Key words Neural precursor cells (NPCs), Astrogliosis, Lentiviral vectors, Neurospheres, Neurogenic molecules, Cortical trauma

1 Introduction

The central nervous system (CNS) can be damaged by a wide range of conditions including infections, hypoxia, stroke, chronic degenerative diseases, and acute trauma. Brain or spinal cord damage results in loss of specific populations of neurons and/or glial cells and is accompanied by the development of defined psychiatric or neurological symptoms of varying severity. Traumatic brain injury (TBI) in particular remains one of the leading causes of mortality and morbidity worldwide; yet despite extensive efforts to develop neuroprotective therapies for this devastating disorder there have been no successful outcomes in human clinical trials to date. This is mainly due to the fact that the adult brain has limited ability to regenerate lost neural tissue after brain damage, partly due to lack of a resident population of neural stem/progenitor cells (NPCs) responsive to injury-derived signals. Limited

compensatory cortical neurogenesis has been reported following stroke [1] or induced apoptotic degeneration [2], but the number of neurons produced is insufficient to replenish neuronal loss after injury and restore cortical function [3, 4]. To overcome this limitation, efforts have been made to stimulate the endogenous NPC population residing in the neighboring subventricular zone (SVZ) with growth factors, in order to recruit a population of NPCs to the lesioned cortex. Yet, adequate recruitment of SVZ NPCs to successfully replace damaged cortical neurons has so far not been achieved [5–7]. In this context, strategies involving transplantation of specific cell populations to replace the damaged cells is a promising approach. Stem cells offer a unique opportunity for the development of cell-based therapies and such prospects have been reinforced since the discovery that adult human differentiated cells may be reprogrammed to a pluripotent embryonic stem (iPS) cell-like state. In view of the heterogeneous nature of the clinical situation in TBI, numerous animal models of such injury have been developed. Although larger animals are closer in size and physiology to humans, rodents are mostly used in TBI research owing to their modest cost, small size, and standardized outcome measurements, among other reasons. More recent TBI models have been targeted at improving our understanding of the detrimental, complex molecular cascades that are initiated by head trauma [8–12]. However, all current animal models mimic some, but not all, types of human brain injury. Achieving a therapeutic breakthrough in TBI will probably require a multifaceted approach combining the following: innovations in clinical trial design, development of new clinically relevant models, refinements of established models and functional tests, consideration of systemic insults and multimodality monitoring, searching for specific and sensitive biomarkers, and optimization of therapeutic dosing and timing of single and combination treatments.

1.1 Brain Injury and Microglia/ Astroglia Activation

Trauma to the brain results in rupture of the BBB, enabling recruitment of circulating neutrophils, macrophages, and lymphocytes to the injured site. The accumulation of blood-borne immune cells within the brain parenchyma has been reported in human TBI as well as animal models of brain trauma [13]. These cells release inflammatory mediators that mobilize glia and immune cells to the site of injury. In addition to the infiltration of immune cells, the activation of resident microglia plays a major role in the response to brain injury [14]. Microglial cells constitute the resident macrophage population of the CNS and transform to an activated state in response to injury. Recent *in vivo* studies using multiphoton microscopy have taken advantage of transgenic mice in which all microglia are fluorescently labelled by expressing the green fluorescent protein (GFP) in the Cx3cr1 chemokine receptor locus [15]. It has thus been revealed that following a laser-induced injury

microglial cells rapidly proliferate and migrate towards the site of injury in response to extracellular ATP released by the injured tissue [16, 17]. The microglial processes then fuse to form an area of containment between healthy and injured tissues, suggesting that microglia may represent the first line of defense following traumatic injury [17]. However, when microglia become over-activated or reactive they can induce detrimental neurotoxic effects by releasing multiple cytotoxic substances, including pro-inflammatory cytokines (e.g., interleukin (IL)-1b, tumor necrosis factor- α (TNF α), and interferon- γ (IFN γ)) and oxidative metabolites (e.g., nitric oxide, reactive oxygen, and nitrogen species) [18].

The release of pro-inflammatory cytokines and other soluble factors by activated microglia significantly influences the subsequent activation of astrocytes and glial scar formation under pathological conditions including central nervous system (CNS) injury [19–21]. Astrocytes react by multiple and complex changes in their morphology, gene expression, and function, a process referred to as “astrogliosis.” Astrogliosis is characterized by cellular hypertrophy [22] and by an increase of intermediate filaments (vimentin and GFAP). In most cases a proportion of reactive astrocytes also start proliferating to seal off the injured tissue and restrict inflammation and neuronal death [23–25]. Hypertrophic astrocytes surround the lesion site and deposit an inhibitory extracellular matrix including chondroitin sulfate proteoglycans that contributes to the glial scar. This dense physical and chemical barrier inhibits axonal regeneration and prevents functional connections required for axonal growth and repair [26]. Upon their activation, astrocytes upregulate a number of neurotrophic factors [e.g., brain-derived neurotrophic factor (BDNF)] that support and protect against injury-induced cell death [27]. In addition, as astrocytes play a crucial role in regulating extracellular glutamate levels, astrogliosis results in reduced glutamate excitotoxicity to neurons and other cells [28]. On the other hand, impaired astrocyte performance exacerbates neuronal dysfunction following brain injury and transgenic ablation of reactive astrocytes increases neuronal cell death and promotes worse outcome after TBI [29]. Collectively, astrocytes provide neurotrophic support and guidance for axonal growth following CNS injury, while on the other, prolonged astrogliosis inhibits axon regeneration and hinders functional recovery. In fact, improved axonal growth and repair following experimental brain and spinal cord injury has been demonstrated in transgenic mice deficient in both vimentin and GFAP [30, 31].

1.2 NPC Transplantation for Repair of Brain Lesions

Transplantation of suitable cell types into the injured brain has attracted considerable interest as a promising strategy to overcome its regenerative limitations [32, 33]. To this end, NPC transplantation has opened new venues for potential therapeutic interventions for neurodegenerative diseases and brain injury. NPCs have been

isolated from embryonic, postnatal, or adult brain tissue of different species [34, 35]. It is well established that these cells consist of a heterogeneous population of mitotically active, self-renewing progenitor, or immature precursor cells that can be expanded in vitro and, under specific conditions, give rise to neurons, astrocytes, and oligodendrocytes [36, 37]. Up to now an increasing number of studies [38, 39] have demonstrated the potential of using NPC for repair of the injured brain and have set the stage for additional approaches involving NPC transplantation in combination with ex vivo gene transfer for expression of regeneration-promoting molecules in the lesioned CNS. This dynamic cell population has been extensively used as a cellular transplantation source in animal models of brain injury, aiming at multiple targets: to replace cells at the injury site (e.g., neuronal replacement), repair the damaged cells (i.e., remyelination), or to alter the local environment to be more conducive for regeneration (i.e., trophic support). The transition of NSCs into the clinical setting, however, is hindered by the fact that the adult CNS does not provide an optimal milieu for exogenous NPCs to survive, engraft, differentiate, and integrate into host tissues. To overcome this limitation several engineering strategies have been developed to improve transplanted cell viability, as well as host tissue integration and targeted differentiation. These include use of biomaterials for creating a regeneration-friendly niche [40, 41] and development of improved vector systems for gene delivery of molecules enhancing the survival stem cells and NPCs [42], or influencing their differentiation towards the appropriate brain cell type. To this direction trophic factors such as NGF [43], GDNF [44], EGF [45], and FGF2 [6], cytokines such as erythropoietin [46], but also cell adhesion molecules [47, 48] and extracellular matrix proteins including tenascin-R [49] have been used to enhance the regenerative capacity of the injured CNS. In these paradigms, introduction of each individual factor resulted in improvement of either cell survival or migration or an increase in the differentiation potential of the transplanted cells towards a desirable pathway. Here, we describe a protocol [50], which involves transplantation of NPCs, genetically engineered to overexpress a neurogenic protein, that have the potency to differentiate with higher frequency towards the neuronal lineage in a rodent model of a stab wound cortical injury (Fig. 1).

2 Materials

2.1 Embryonic Neurosphere Culture and Lentiviral Transduction

1. E14.5 actin-GFP transgenic mice.
2. DMEM/F12 medium.
3. N2 supplement.

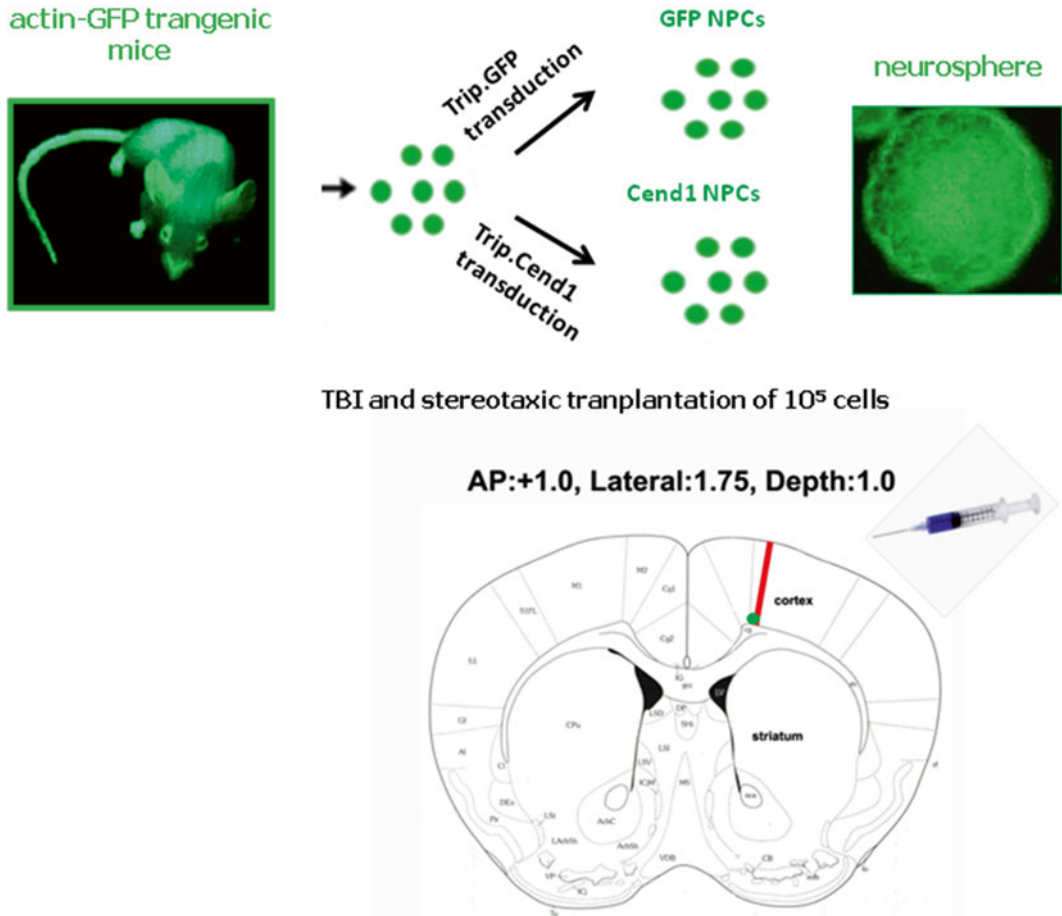


Fig. 1 Schematic representation of the production and viral transduction of NPCs from actin-GFP mice and diagram illustrating the injury site (*red line*) in the motor cortex area and NPC grafting (*green dot*)

4. 20 μ g/ml Insulin.
5. 20 ng/ml recombinant human basic fibroblast growth factor (bFGF).
6. 20 ng/ml Epidermal growth factor (EGF).
7. Nucleospin tissue kit.
8. Quantitative SYBR Green PCR kit.

2.2 Traumatic Brain Injury and NPC Transplantation

1. Female C57BL/6J mice 2–3 months old.
2. Imalgene (100 mg/ml, 0.03 ml/20 g body weight).
3. Rompun (20 mg/ml, 0.005 ml/20 g body weight).
4. Stereotaxic apparatus for mice.

5. 26-gauge needles.
6. 5-Bromo-2-deoxyuridine.

2.3 Immunohistochemistry, Quantification, and Statistical Analysis

1. 4 % Paraformaldehyde (PFA) in PBS solution.
2. Vibrating microtome.
3. PBS/0.2 % gelatin/0.2 % Triton X-100.
4. 2 N HCl/0.2 % Triton X-100.
5. 0.1 M Sodium borate, pH 8.5.

2.4 Primary and Secondary Antibodies

1. Rat polyclonal anti-BrdU (1:100; Sigma), to detect proliferating cells.
2. Chicken polyclonal anti-nestin (1:100; Novus Biochemicals) to detect NPCs.
3. Mouse monoclonal anti-nestin (1:100; Chemicon) to detect NPCs.
4. Mouse monoclonal anti-neuronal class β III-tubulin (1:400; Covance) to detect neurons.
5. Mouse monoclonal anti-NeuN (1:100; Chemicon) to detect neurons.
6. Rabbit polyclonal anti-GFAP (1:700; Dako) to detect astrocytes.
7. Mouse monoclonal anti-O4 (1:400; Chemicon) to detect oligodendrocytes.
8. Rabbit polyclonal anti-phospho-histone H3 (pH3) (1:300; Upstate) to detect mitotic cells.
9. Rabbit polyclonal anti-caspase-3 (1:100; Cell Signaling) to detect apoptotic cells.
10. Rabbit polyclonal anti-GABA (1:100; Sigma) to detect GABAergic interneurons.
11. Rabbit polyclonal anti-glutamate (1:100; Sigma) to detect glutamatergic neurons.
12. Mouse monoclonal and rabbit polyclonal AlexaFluor 546 (red) or 647 (blue) secondary antibodies (Molecular Probes).
13. TO-PRO-3 (1:1,000; Molecular Probes), for nuclear staining.
14. Superfrost slides.
15. Mowiol* 4-88 reagent (Calbiochem).
16. TCS SP confocal laser scanning microscope (Leica).
17. Image-Pro Plus image analysis software.

2.5 Behavioral Assessment

Rotarod apparatus for mice equipped with an automatic fall detector.

3 Methods

All experiments are carried out in room temperature, unless otherwise specified.

3.1 Embryonic Neurosphere Culture and Lentiviral Transduction

1. Embryonic neurospheres (NPCs) are derived from the cortical ventricular zone (VZ) of E14.5 transgenic C57BL/6J mice expressing the green fluorescent protein GFP (GFP-NPCs) under the control of the ubiquitous beta-actin promoter (actin-GFP mice) [51].
2. VZ is dissected and mechanically dissociated by several passages through a fire-polished glass pipette.
3. The resulting single-cell suspension is placed in neurosphere medium consisting of DMEM/F12 medium supplemented with N2, insulin, recombinant human basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) and cultured in 6-well plates (3 ml/well) in a cell incubator at 37 °C, 5 % CO₂, and 95 % humidity.
4. After 2–3 days in culture small-size free-floating neurospheres start to form.
5. After 7 days in culture, when neurospheres have acquired a significantly bigger size and higher density, they are single cell dissociated and allowed to re-form spheres at least four times before further use (*see Note 1*). During the culture period the neurosphere medium is changed by half every 2–3 days and supplemented by freshly made one.
6. For lentiviral transduction, fourth-passage neurospheres get single cell dissociated, grown in suspension for 2–3 days and then incubated at a total volume of 100–200 µl with the Trip.Cend1 or control Trip.GFP viral vectors for 7 h diluted 1:1 in neurosphere medium (*see Note 2*).
7. Viral transduction efficiency is estimated after 3 days both by monitoring transgene Cend1 expression by immunofluorescence labelling and by real-time PCR to evaluate viral copy number using a known lentiviral backbone sequence.
8. The viral copy number of Trip.Cend1 and Trip. GFP-transduced neurospheres subsequently cultured for short (3 days) and long (1 and 2 passages, 10–20 days) time periods (*see Note 3*) are analyzed by quantitative SYBR Green PCR in genomic DNA samples purified using Nuclospin tissue kit.7 (*see Note 4*).
9. All DNA samples are analyzed in duplicate 20 µl reactions. Standard curves are plotted using the threshold cycle (Ct) values of limited dilutions of control pTrip plasmid (10⁹–10² copies/µl) and NIH-3 T3 mouse cell DNA (0.025–125 ng). These are then used to extrapolate the absolute lentiviral copy number and number of cells per reaction, respectively.

3.2 Traumatic Brain Injury and NPC Transplantation

1. Female C57BL/6J mice 2–3 months old are deeply anesthetized intraperitoneally with Imalgene and Rompun (*see Note 5*) and positioned in a stereotaxic frame.
2. A burr hole is drilled at coordinates AP 1.0, L 1.75 (relative to Bregma = 0) using a dental drill and stab wound injury is caused to the right hemisphere of the cerebral cortex by inserting a 26-gauge needle 1.0 mm deep from the brain surface (DV 1.0 relative to Bregma = 0) taking care not to injure the subcortical white matter. The needle is then retracted and reinserted twice.
3. Immediately after injury 1 μ l of freshly dissociated GFP-NPCs from actin-GFP mice (10^5 cells), either transduced with Trip. Cend1 (Trip.Cend1-GFP-NPCs) or non-transduced (GFP-NPCs), is injected 0.3 mm ventrally to the injury site using a 1 μ l syringe with a 26-gauge needle.
4. Cells are injected slowly over 5 min, the syringe is left in place for an extra 5 min and then being withdrawn gently (*see Note 6*), and the skin is sutured.
5. After surgery mice are held on a heated cushion before being returned to their home cages. Lesioned animals receive 5-bromo-2-deoxyuridine (BrdU) in their drinking water during survival time [52] and get sacrificed 1 or 4 weeks after the operation.

3.3 Immunohistochemistry, Quantification, and Statistical Analysis

1. Operated mice are transcardially perfused with 50 ml PBS followed by 50 ml 4 % PFA and their brains are removed and post-fixed overnight at 4 °C in the same fixative.
2. Each brain hemisphere is embedded in 4 % agarose and 40 μ m thick coronal sections are cut in a vibrating microtome and processed for immunohistochemistry.
3. Free-floating vibratome sections are incubated for 30 min at 37 °C in PBS/0.2 % gelatin/0.2 % Triton X-100. For BrdU detection, sections are treated with 2 N HCl/0.2 % Triton X-100 for 15 min followed by 0.1 M sodium borate, pH 8.5, prior to blocking with gelatin and application of anti-BrdU antibody.
4. Sections are incubated overnight at 4 °C with primary antibodies in PBS/0.02 % gelatin/0.2 % Triton X-100.
5. Following primary antibodies, sections are washed twice in PBS and incubated for 2 h with the appropriate secondary antibodies conjugated with AlexaFluor 546 (red) or 647 (blue) diluted 1:500 in PBS/0.02 % gelatin/0.2 % Triton X-100.
6. At the end of immunofluorescence labeling, cell nuclei are stained with TO-PRO-3 (1:1,000 in PBS) for 5 min.
7. Sections are mounted on Superfrost slides using Mowiol and covered with cover slips (*see Note 7*).

8. To quantitate the degree of proliferation or apoptosis, and the neuronal or the glial differentiation of grafted NPCs, immunostained sections are analyzed by confocal microscopy. Confocal images of immunofluorescence labeling are obtained using a 40× lens and constant image acquisition procedures.
9. The red or the blue channel of the images, corresponding to cell type-specific marker immunolabeling, are used for computer-assisted analysis in combination with the green channel corresponding to GFP fluorescence of the grafted cells, utilizing the Image-Pro Plus image analysis software. Only the marker-positive fluorescence overlapping with the green GFP signal is taken into account. The ratio of marker fluorescence overlapping with the GFP signal over the total GFP signal is calculated and used as an estimate of the percentage of marker⁺/GFP⁺ grafted cells out of the total number of GFP⁺ grafted cells.
10. The extent of astrogliosis in the lesioned hemisphere is also measured at different time points following transplantation as follows: all sections of the right hemisphere encompassing the lesioned area along the rostro-caudal for a total number of three animals per group are immunostained using antibodies to glial fibrillary acidic protein (GFAP).
11. Confocal images of all GFAP-immunostained sections are obtained using a 10× lens and constant image acquisition procedures. The red channel corresponding to GFAP immunofluorescence is then used for computer-assisted analysis with the help of the Image-Pro Plus image analysis software. The GFAP⁺ area around the injury site is marked taking care not to include the subcortical white matter, and GFAP fluorescence is quantified within this area. In all transplantation experiments statistical analysis is performed by the Student's *t* test.

3.4 Behavioral Assessment

A Rotarod apparatus for mice is used to measure motor coordination and balance [2].

1. Prior to surgeries, mice are trained on the Rotarod wheel for three consecutive days (three trials per day), running at an accelerating speed increasing from 4 to 40 rpm over 5 min.
2. Initial Rotarod tests are performed 24 h before injury to record baseline latencies for each animal. Three trials for slow (16 rpm) and three for fast (32 rpm) speed are performed up to 3 min and the best performance value for each animal is recorded. Animals that fail to stay on the Rotarod for >120 s at 16 rpm are excluded from the experiment. A trial is terminated if the animal falls off the Rotarod or grips the device and spins around past the lowest point.

3. Post-injury testing is monitored once a week for a total period of 4 weeks. Statistical analysis is performed using ANOVA followed by Tukey's post hoc analysis with multiple comparison.

4 Notes

1. Neurospheres should be re-dissociated and allowed to re-form spheres at least four times before viral transduction and further use, in order to enrich the neural stem cell population in the culture.
2. The size of neurospheres and the time duration of their incubation with the viral vectors are critical for transduction efficiency. For optimum transduction efficiency, neurospheres should be relatively small (2–3 days following single-cell dissociation) and incubation with the viral vectors should be performed in minimal volume (no more than 200 μ l total volume) for no longer than 8 h, to reduce cell death caused by the viral toxicity.
3. The number of viral copies in transduced cells should be also figured out after several passages of neurospheres following viral transduction (long periods), to ensure that the viral vectors have been inserted into cell's genome and do not remain episomal.
4. In this protocol a lentivirus-based gene transfer system [53] can be used to overexpress lineage-specific regulators and thus control cell cycle and differentiation properties of cortical NPCs in vitro. At a next step, genetically modified NPCs are stereotactically transplanted in a mouse model of cortical stab wound injury and their differentiation properties into the tissue are monitored. Using this protocol we have reported that genetically modified NPCs overexpressing Cend1 show increased neuronal differentiation in vivo and generate a larger proportion of neuronal cells after grafting, as compared to control NPCs. Additionally, Cend1 overexpressing NPCs reduce astroglial scar formation around the injury site. The observed effects of Cend1 overexpression in grafted NPCs may be beneficial for repair of brain lesions [50].
5. Weighing each animal before operation is critical to estimate the exact dose of anesthesia needed and avoid lethality.
6. NPCs should be injected slowly within the tissue and the syringe should be left within in place for an extra 5 min, in order to avoid spill off and ensure that no cells have remained within the syringe.
7. Care should be taken to avoid air bubbles while mounting the vibratome sections onto slides and covering with cover slips. No mechanical pressure should be introduced over sections and they should be left for 12 h in RT before imaging, allowing Mowiol to fully solidify.

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Experimental Cell Transplantation for Traumatic Spinal Cord Injury Regeneration: Intramedullar or Intrathecal Administration

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Abstract

Animal experimentation models are a necessary prerequisite to human trials for the use of regenerative medicine in the treatment of spinal cord injuries. Considerable effort is required for the generation of a consistent and reproducible methodology to incur an injury and evaluate the results. The traumatic contusion model has been accepted as a model that closely mimics a typical human traumatic injury, and here we detail step by step an approach to generate a reproducible lesion in rats. Acute cell transplantation by intramedullar or intrathecal administration is described for regenerative interventions. The same model is suitable to design subacute or chronic therapeutic approaches by interventions 1 week or 1 month after lesion.

Key words Spinal cord injury, Rat, Cell transplantation, Traumatic lesion, Intramedullar, Intrathecal

1 Introduction

Considerable research has been performed in the last 20 years using experimental models to detail the spinal cord injury (SCI) process and to apply potential therapeutic tools, in many cases with therapeutic success [1–3]. Nevertheless, neuroscientists agree that often there is a problem of reproducibility of results from different labs, largely due to technical difficulties [4]. Although regenerative medicine-related research has been performed for the last few decades with therapeutic success, a large gap still exists between experimental data and clinical practice. Recent studies demonstrate the potential of certain cell therapies to incorporate new neural cells into the milieu of traumatic spinal cord injury, regenerate or remyelinate axons providing new oligodendrocytes, or simply reconnect injured tissue with newly generated neurons [2, 3, 5–10].

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Rats are the most common mammalian models for SCI experimentation. Rats maintain equilibrium between adult body weight, life-span, and anatomical organization of the central nervous system allowing one to manage a considerable number of individuals at the same time. Contusive injuries are thought to be more similar, in terms of biomechanical and inflammatory components, to injuries seen in the clinic, thereby providing the most realistic experimental setting in which to test potential neuroprotective strategies.

The question of “how” and “when” to perform cell transplantation is a critical one. Acute approaches have shown more promising results than treatment of chronic lesions marked by the potential effect of neuroprotective versus regenerative mechanisms [11]. Subacute transplantation allows for better cell survival and avoids the initial inflammatory response, which is highly toxic to transplanted cells [12, 13]. Acute intramedullar transplantation represents a more accepted proof-of-concept approach in order to minimize the surgical procedure. However, intrathecal cell administration is becoming more popular due to its suitability with regard to clinical translation, less invasive nature, and reduced trauma for the spinal cord tissue surrounding the injured area, with higher impact in subacute or chronic transplantations.

Each SCI in humans features a different lesion, while lesions in the laboratory have to be made as reproducible as possible for consistent data. Here we present a protocol with different cell therapy approaches in a contusive traumatic spinal cord injury model which is more similar to that seen in a clinical scenario. These are suitable to examine acute, subacute, or chronic cell transplantation and to investigate neural and functional repair of the damaged tissue by the exogenous transplanted cells [14, 15].

Cell replacement and in particular stem cell transplantation is a very promising strategy that allows bridging the lesion site either in the acute or in the chronic phase. The implanted cells could create an environment in which remyelination, axon elongation, and formation of new circuits may occur as it has been probed in rodents where the functional analysis and cell and molecular mechanisms involved can be closely analyzed. Different types of cells, including human origin, have shown regenerative potential including neural precursors [5, 8], oligodendrocyte precursor cells [5], or mesenchymal cells [16]; however, a reproducible, detailed protocol for surgery and cell transplantation procedure has not yet been described.

2 Materials

2.1 Equipment/Small Apparatus

1. DKI 900 Small Animal Stereotaxic Instrument mounted on DKI 980 Rat Spinal Unit (David Kopf Instruments, CA, USA).
2. DKI 5000 Microinjection Unit (incl. 5001 Holder) (David Kopf Instruments, CA, USA).

3. Nanomite, Infuse/Withdraw.
4. 25 µl Hamilton syringe.
5. IH Spinal Cord Impactor (Precision Systems and Instrumentation, LLC).
6. Anesthesia workstation.
7. Rat anesthesia mask.
8. Heating pad.
9. Hot glass bead dry sterilizer.
10. Surgical shavers for rodents, ISIS.
11. Infusion syringe pumps.
12. Gas absorber (CA-AG1000, Cibertec, Spain).

2.2 Surgical Tools

1. Scalpel #3.
2. Blades.
3. Scissors, blunt/blunt, straight.
4. Scissors, straight sharp/blunt.
5. Forceps, serrated Adson (No. 11006-12, FST, USA) (2×).
6. Forceps, fine sharp teeth Adson Graefe (No. 11030-12, FST, USA).
7. Rongeur: Fridman-Person (Straight, No.16220-14, FST, USA).
8. Fine iris scissors, cutting edge 8 mm.
9. Spinal Cord Hook (No. 10162-12, FST, USA).
10. Double Pronged Pick (No. 18067-11, FST, USA).
11. Retractor: Alm retractor (No. 17008-07, FST, USA) (2×).
12. Needle holder.
13. Micro-mosquito, straight.
14. Micro-mosquito, curved.
15. 986B Vertebrae Clamp (Rat).
16. Double-ended micro-spatula.
17. Austin Chisel.
18. Stainless steel sterilization container.
19. Bone wax hemostats.
20. Monosyn violet 4/0 HR26.
21. Intravenous cannula with fixation wing.
22. 1 ml syringes.
23. 25 G needles.
24. Cotton swabs and gauze.
25. Polyurethane rat intrathecal catheter.
26. Headband magnifier visor.

2.3 Reagents

1. Isoflurane.
2. 0.2 % Lipolac.
3. Betadine solution.
4. 1 % Chlorhexidine.
5. 2 % Lidocaine.
6. Buprenorphine.
7. Enrofloxacin.
8. Solid drink.
9. Cyclosporine.
10. Morphine.
11. 0.9 % NaCl saline solution.
12. 3 % Hydrogen peroxide.
13. Trypan blue.
14. Deoxyribonuclease I.

2.4 Experimental Individuals

Adult female rats, 2 months old, 200 g of body weight: Food and water provided ad libitum during the entire experiment. All surgical procedure steps have to be performed according to ethical procedures for the use of animals in laboratory experiments. The experimental protocol used here was approved by the Animal Care Committee of the Research Institute Principe Felipe (Valencia, Spain) in accordance with the National Guide to the Care and Use of Experimental Animals (Real Decreto 1201/2005).

2.5 Cell Suspension

Typically 1×10^6 cells at a concentration of $1 \times 10^5/\mu\text{l}$ are used. Under sterile conditions and after regular homogenization (mechanical or enzymatic) for single-cell suspensions, centrifuge the mixture several times to completely remove any residual enzymatic activity. Quantify the cell viability by trypan blue exclusion analysis and if necessary disrupt any cell aggregate by using glass pulled pipettes (*see Note 1*). Often a percentage of cell death and release of DNA occurs increasing the viscosity of the cell suspension, so it is recommended to incubate for 5 min at 37°C with DNase I (125 U/ml) and then wash by centrifugation. If different species are used for hosting the donor cells immunosuppressant treatment is needed (*see Note 2*). Finally, add the appropriate volume of growth medium to reach the desired cell concentration. Keep the cell suspension on ice until use.

3 Methods

3.1 Presurgical Procedure

1. Sterilize all small surgical instruments in a stainless steel sterilization container by regular humid autoclave ($121^\circ\text{C}/20\text{ min}$). Large instruments and equipment can be cleaned with 70 % ethanol.

Table 1
Drug administration during surgical procedures

Stage for treatment	Desired effect		Active principle	Administration
Presurgery	Analgesic Antibiotherapy		Morphine sulfate Enrofloxacin	1.1 mg/kg b.w/4 h 12 mg/kg b.w/day
Intrasurgery	Analgesia	Induction Maintenance	Buprenorphine	0.05 mg/kg b.w/20 min
	Local anesthesia		Lidocaine 2 %	2 %/h
	General anesthesia		Isoflurane	3 %/continuous infusion
			Isoflurane	1.5–2 %/continuous infusion
	Fluidotherapy	Physiologic saline solution 0.9 % NaCl	NaCl	0.9 %/0.2 ml/h
	Eye lubricant		Polyacrylic acid	1 drop/eye
Postsurgery	Analgesia		Buprenorphine	0.1 mg/kg b.w/12 h
	Antibiotherapy		Enrofloxacin	12 mg/kg b.w/day
	Dehydration recovery		Solid drink	10 ml bag/day
	Ulcer prevention/ treatment	Prevention	Hyperoxygenated acids, <i>Hypericum perforatum</i>	1 time/day
		Treatment	Asiatic Centella, neomycin sulfate	2 topic applications/day
			Bacitracin, neomycin sulfate, polymyxin B sulfate	2 topic application times/day
		Intestinal transit activation		Liquid paraffin (4 g/5 ml)

2. For antibiotic prophylaxis and analgesic, subcutaneously inject the antibiotic (enrofloxacin) and morphine solution 30 min prior to the surgical procedure as indicated in Table 1.
3. Mount the heating pad, stereotaxic instrument, anesthesia mask, and spinal cord (Fig. 1a).
4. Set up the anesthesia workstation and fill with isoflurane, including the connection to the gas absorber.
5. Set up the instruments on the surgical table (Fig. 1b).
6. Switch on the IH Spinal Cord Impactor and open a new window in the operator software.

3.2 Surgery Procedure

1. Rat induction for deep anesthesia plane with 3 % isoflurane using the plexiglass chamber connected to the anesthesia workstation (Fig. 1c).
2. Move the animal to the anesthetic mask, with 1.5–2 % isoflurane, when it has been anesthetically induced (normally 1–2 min after 3 % isoflurane induction) and check the anesthesia stage;

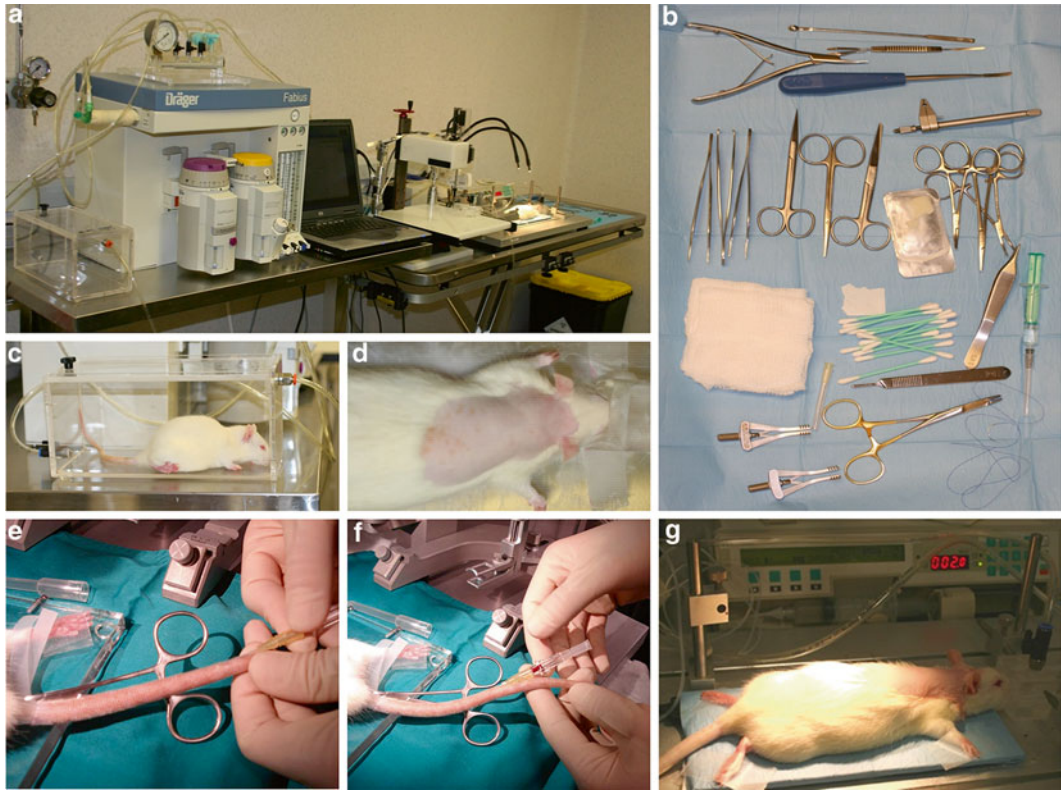


Fig. 1 Presurgical setup

the muscles should be relaxed, with no pedal retraction or palpebral or corneal reflexes (Fig. 1d).

3. Shave the dorsal area between the neck and hindlimbs extending ~2 cm bilaterally from the spine and spray with chlorhexidine solution covering the whole shaved area and paint the surgical area with betadine (Fig. 1d). It is recommended to use a separate surface for shaving the animals in order to avoid contamination in the surgical area.
4. Position the animals on a heating pad (37 °C) mounted on the spinal cord unit with stretched anterior and posterior legs and adjusting the mouth and nose into the anesthetic mask while keeping enough space for gas interchange (Fig. 1g).
5. Set up the anesthesia workstation at 1.5 % of isoflurane and maintain this flow for the duration of the surgery.
6. Introduce an intravenous cannula in the more visible and caudal vein in the tale (Fig. 1e, f). Connect the pre-filled cannula to the continuous 0.9 % of NaCl perfusion, 2 ml/h, and maintain this caudal during all surgical and transplantation procedure.
7. Moisten each eye by using the eye drops, 1 drop for each eye, and then close the eyes.

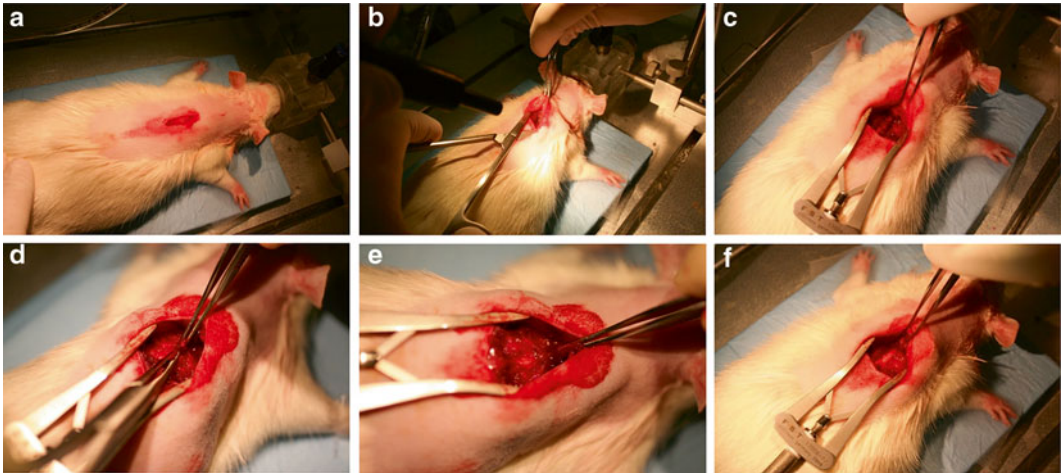


Fig. 2 Dorsal laminectomy

8. Perform a longitudinal skin incision of approximately 2.5 cm with scalpel blade (Fig. 2a).
9. Dissect the fat tissue without cutting it, keeping the fat pad under the skin (Fig. 2b).
10. Make an incision on the middle line of the muscles overlying the vertebral column exposing the T7–T10 vertebral segments (Fig. 2c).
11. Position the alm retractors to keep the incision widely open. It is very important to visualize the thoracodorsal artery, located over the T6 segment but avoid touching it, or risk a hemorrhage complication.
12. Detach the spinotrapezius muscle from bone on the spinal laminae using the scalpel blades or detacher. Use the headband magnifier visor for fine visualization of the operation procedure.
13. Identify the T7 and T8 vertebral apophysis by anatomical criteria (both are transversal and parallel to each other; Fig. 3).
14. Under the headband magnifier carefully lift the T9 spine backwards while introducing slowly a rongeur of a very fine-pointed side-cutting bone. Cut out the T9 and T8 vertebral apophysis leaving clean lateral spaces and avoiding lateral compression of the cord (Fig. 2d, f).
15. Carefully move the rat into the impactor equipped with an additional anesthesia mask (Fig. 4a–c). Fix the T7 and T10 lateral vertebral apophysis with the forceps linked to the impactor device keeping the cord well stabilized and in a parallel plane. By applying a 100–250 kdyn force the computerized device will generate, respectively, a moderate-to-severe impact.

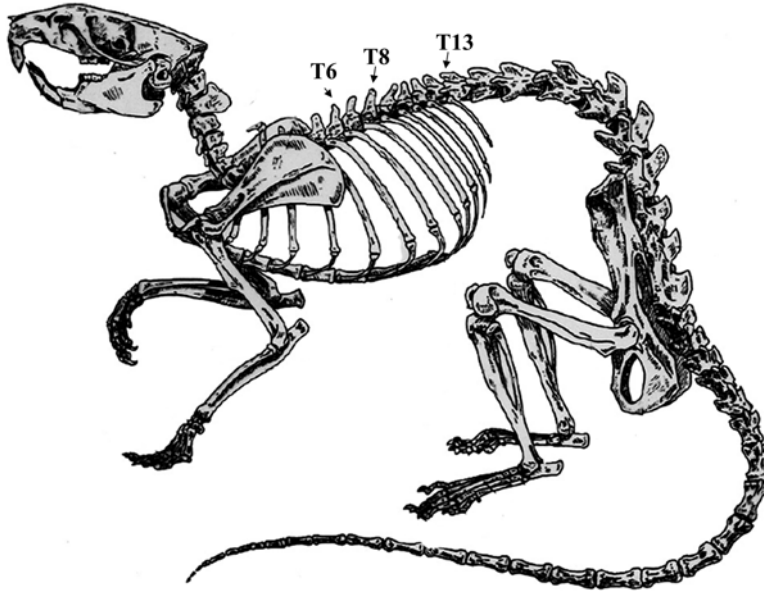


Fig. 3 Anatomical view of thoracic spinal segment

This fall is monitored by multiple sensors connected to a computer that records the weight's height of fall and speed of fall, as well as the instant of impact with the dura mater, the change of position of the spinal cord after the impact, and the spinal cord compression caused by the fall. It is important to maintain the conditions for reproducible contusions. Records of the impact magnitudes and tissue displacement indicated no significant differences in impact parameters between groups that might account for behavioral differences. Normally a transversal hematoma on the impact region is visualized and the cord rapidly swells (Fig. 4d). Addition of local lidocaine in the impacted spinal cord is optional (*see Note 3*).

16. Move the animal back into the surgical mask and proceed to cell transplantation. *See Note 4* for variants on the cell transplantation procedure.
17. For intramedullary cell injection: Immobilize the spinous processes using vertebral clamps fixed to a spinal cord unit securing the vertebral column T10 (Fig. 5a, b).
18. Prepare cells as described in Subheading 2.5.
19. Position the Hamilton pipette filled with cell suspension mounted into the microinjector, nanoliter device, on the spinal cord surface and inject the cells 2 mm caudally and 2 mm rostrally to the transversal hematoma produced by the impactor. The host spinal cord cranial and caudal to the lesion epicenter is targeted to avoid the epicenter cavitation, hemorrhagic

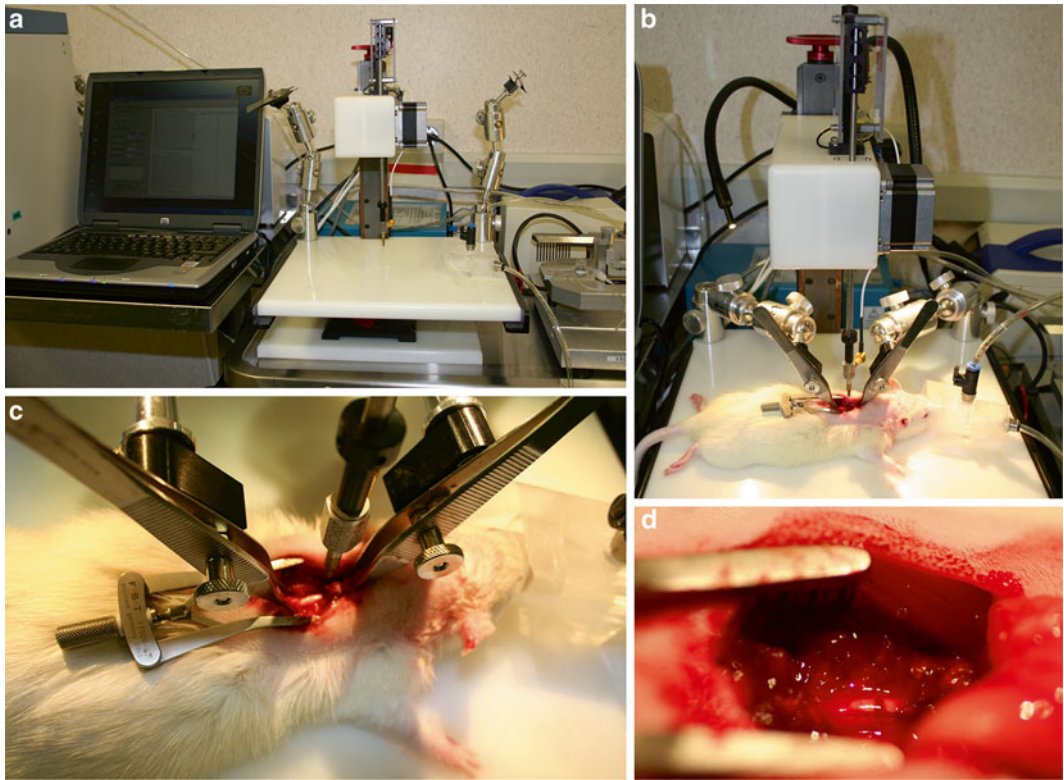


Fig. 4 Spinal cord injury by traumatic contusion

necrosis, and inflammation, and to target the penumbra of the lesion, which might decrease cell survival and integration.

20. Deliver the cells, contained into 5 μ l, from ventral to dorsal column in two points no faster than 2 μ l/min. Wait for 1 min between injections before moving the syringe to allow cell deposition into the medullar tissue (Fig. 5d).
21. For intrathecal cell administration: Partial laminectomy of T13 (previous external incision and muscle retraction are performed similarly to previously described in **steps 8–12** and Fig. 6a, b) allows introducing the catheter (previously filled with 0.9 % saline solution) through a hole in the dura mater with a 22 G needle, up to the injured segments (T8) (Fig. 6c, d). Push up the catheter very slowly to avoid additional damage. Normally the catheter is visualized within the injured area under the dura mater. Erection of the hair and automated reflex in the legs are signs of correct location of the catheter into the intrathecal space. In addition, the catheter is filled by capillary absorption of cerebrospinal fluid, often contaminated with blood due to the hemorrhage after contusion (Fig. 6d).

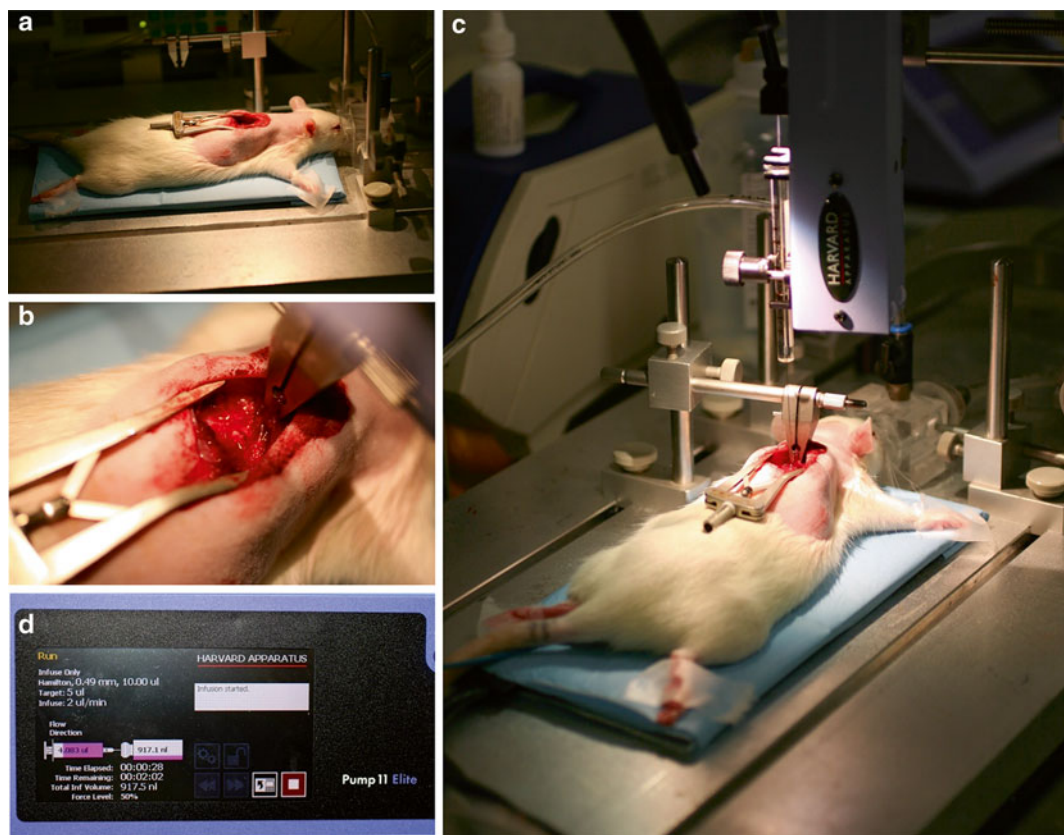


Fig. 5 Intramedullar cell transplantation

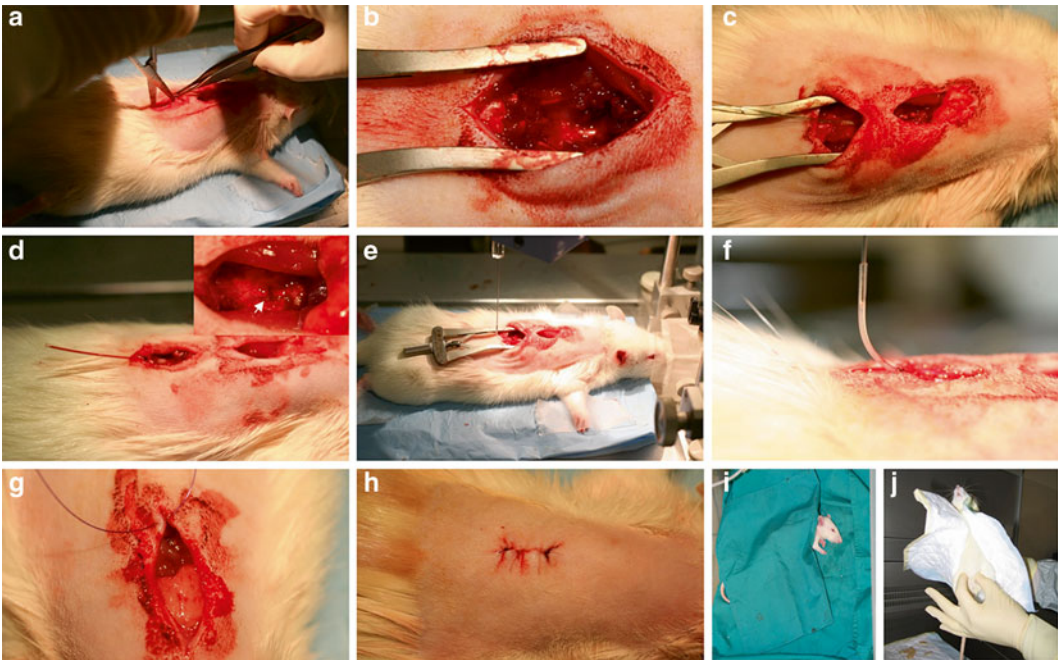


Fig. 6 Intrathecal cell transplantation

22. Cut the extra tube and connect the end of the catheter to the Hamilton syringe previously filled with the cell suspension (prepared as previously described in Subheading 2.5).
23. Deliver the cells, 5–10 μ l, into the injured area by administration of 2 μ l/min (Figs. 5d and 6e, f). Wait for at least 1 min before retracting the catheter.
24. Control animals should be injected by intrathecal catheter with the same volume of cell medium.
25. Cover the laminectomy areas with a piece of subcutaneous fat pad.
26. Remove vertebrae clamps and retractors.
27. Carefully suture the deep and superficial muscle layers with absorptive Monosyn 4/0 and finally suture the skin (Fig. 6g, h).
28. Remove the superficial blood on the incision area with diluted H_2O_2 .
29. Close anesthesia flow and leave animal to wake up on a heating pad (Fig. 6i).
30. Carefully empty the animal's bladder by manually pressing the bladder until it is completely empty (Fig. 6j).

3.3 Post-surgery Procedure

1. Four hours after morphine administration, rats are treated with buprenorphine for analgesic purposes as indicated in Table 1.
2. Include a solid drink bag and a few pellets inside the cages, and provide water and food ad libitum.
3. Manually empty the bladders of all experimentally injured rats twice daily until autonomous bladder function is restored within 2–3 weeks post-injury.
4. Inspect the rats for weight loss, dehydration, discomfort, and autophagy, with appropriate veterinary care if needed (*see* Notes 5 and 6). Do not include the rats whose weight has declined more than 20 %.
5. Administer antibiotics (enrofloxacin, Table 1) for 7 days after surgery to prevent possible infections.
6. Perform daily rehabilitation for 15 min consisting of passive mobilization through a full range of movements to maintain joint flexibility and reflexes in the hindlimbs.
7. The open-field locomotor test of Basso, Beattie, and Bresnahan (BBB) [17] (after video recording twice a week) and electrophysiological records (normally evoked potentials [5, 14]) are the main functional test used.

4 Notes

1. It is important to transplant a population of cells with a high survival rate, and discard cell suspensions with survival rates less than 90 %.
2. If immunosuppressant treatment is needed in order to better preserve the allosteric cell transplantation, daily administration of cyclosporine A (20 mg/kg body weight) is needed during the whole experimental procedure.
3. Addition of one drop of local anesthesia, lidocaine, directly into the previous contusion in the spinal cord may reduce the posterior shock, which often involves respiratory depression.
4. If subacute or chronic cell transplantation is desired, 1 week or 4 weeks after traumatic injury, respectively, the animals should be prepared for surgery again to open and access the injured area and proceed to cell transplantation as previously described. In fact, a combination of different approaches is possible, for example, acute intramedullar cell transplantation and then subacute or chronic intrathecal new cell administration.
5. Additional complications due to paralyzed muscles can appear, such as positional related ulcers. In this case is important to prevent the formation of large ulcers by applying Mepentol®. Rapid and proper treatment of ulcers (*see* Table 1) with Blastoestimulina® and or Dermisone® is strongly recommended.
6. During the first days after injury check for signs of defecation because intestinal paralysis can occur. In this case orally administer Hodernal® once a day until intestinal motility recovers.

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Chapter 4

Generation of Murine Xenograft Models of Brain Tumors from Primary Human Tissue for In Vivo Analysis of the Brain Tumor-Initiating Cell

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Abstract

The generation of xenograft models, which support the growth of human tissue in animals, forms an important part of a researcher's tool kit and enhances the ability to understand the initiation and development of cancer in vivo. Especially in the context of the brain tumor-initiating cell (BTIC), a xenograft model allows for careful characterization of BTIC roles in tumor initiation, growth, and relapse. Here, we detail a set of procedures which describe the isolation, enrichment, and intracranial injection of human BTICs from patient samples to generate xenograft models of a human brain tumor.

Key words Flow cytometry, Brain tumor-initiating cell (BTIC), Neural stem cell, Intracranial injection, NOD SCIDs, Xenograft

1 Introduction

The concept of a brain tumor stem cell was first developed in the wake of the discovery of the adult neural stem cell and following the finding of the role of cancer stem cells in hematopoietic diseases, such as leukemia [1, 2]. Several studies demonstrate that a rare fraction of CD133⁺ cancer cells within brain tumors are capable of multi-lineage differentiation, clonal tumorsphere formation in vitro, and serial tumor transplantation in vivo [3–6]. It has been suggested that this stem cell-like fraction, referred to as brain tumor-initiating cells (BTICs), is resistant to current therapeutic modalities and is likely responsible for the relapse observed in many pediatric and adult brain tumors [5, 7, 8]. Consequently, the elucidation of new treatment strategies with specific activity against this population is of significant interest in the drive to reduce relapse rates and mortality in brain cancer patients.

Xenograft mouse models of brain central nervous system tumors have become one of the primary tools to answer a variety of research questions related to the role of the BTIC in an in vivo environment. The models allow for investigation of in vivo BTIC tumorigenicity, interaction of BTIC populations with the surrounding microenvironment/niche, and the role of the BTIC in causing tumor relapse after treatment with conventional chemotherapeutic modalities [6, 9, 7]. Some of the first murine-derived xenograft models of human brain malignancy involved the intracranial injection of brain tumor cells into the brains of both immune-competent and immune-deficient mice [10–12]. However, recent xenograft models typically take advantage of newer immune-deficient mouse strains such as NOD SCID, NRG, and NSG (nonobese diabetic severe combined immuno-deficient, NOD Rag1, NOD SCID IL2 gamma) mice, which carry combinations of mutations in *RAG1/2 VDJ* recombination machinery, *IL2* receptor gamma chain, and the *SCID* gene [13]. The deficiency in immune response increases tumor engraftment efficiencies due to reduced rejection of human tissue by the murine immune system.

Cell surface molecules have become widely used to identify and enrich for cancer stem cell populations. In the context of brain tumor studies, CD133 and CD15 are the most prevalent markers in the field. A five-transmembrane glycoprotein, CD133 remains the single most utilized BTIC surface marker. CD133 was first described as being co-expressed with CD34⁺ hematopoietic stem cells [14]. In the years following, CD133 was used to isolate central nervous system stem cells [15] and BTICs [4]. In the context of cerebellar tumors such as medulloblastoma, CD15 has been shown to mark a population of cells similar to neuronal progenitor cells with tumorsphere and tumor-initiating properties [16]. Although advancements in technology have made cell surface markers a viable option for BTIC isolation, limitations still exist. Environmental conditions, such as hypoxia, and the use of trypsin for tissue digestion can dynamically alter the presence of cell surface markers. In addition, the expression and topography of these markers are in constant flux as the BTIC rapidly evolves over time and passage. Therefore, current methods can be complemented with in vivo serial transplantation assays to verify the isolated BTIC population [17].

Here we describe procedures whereby primary brain tumors, freshly resected from patients, are dissociated into single cells and cultured in conditions that support the growth and enrichment of cancer stem cell populations. If initial culture is successful and cell viability is maintained, the cells can be sorted to enrich for a tumorigenic BTIC population, which is then injected intracranially into an immune-deficient murine host. Finally, we detail the process of xenograft harvest for the purposes of analyzing tumor morphology

through immunohistochemistry (IHC), flow cytometric analysis of brain tumor marker expression, analysis of vascularity or size, or serial passaging.

2 Materials

2.1 Tumor Processing

1. Artificial cerebral spinal fluid (aCSF), 0.124 M NaCl, 5 mM KCl, 32 mM MgCl₂, 262 mM NaHCO₃, 10 mM glucose, 0.0000972 M CaCl₂ pH ~7.5.
2. 0.2 Wunsch U/mL Liberase/Blendzyme 3.
3. Nylon mesh 70 µm cell strainer.
4. Dulbecco's phosphate-buffered saline (PBS).
5. Ammonium chloride solution, 0.8 % NH₄Cl, 0.1 mM EDTA in water.
6. 100 or 60 mm Ultralow attachment culture dish.
7. Neural stem cell (NSC) complete media for 500 mL, 480 mL Dulbecco's modified Eagle's medium/F12, 5 mL N2 supplement, 5 mL 1 M HEPES, 3 g glucose, 1 mL of 60 mg/mL N-acetylcysteine, 10 mL neural survival factor-1. Growth factors and antibiotics added to the media include 20 ng/mL human recombinant epidermal growth factor, 20 ng/mL basic fibroblast growth factor, 10 ng/mL leukemic inhibitory factor, and 1× antibiotic-antimycotic solution.

2.2 Primary Cell Culture

1. NSC complete media.

2.3 Sorting BTICs by Flow Cytometry

1. Dulbecco's phosphate-buffered saline (PBS).
2. 0.2 Wunsch U/mL Liberase/Blendzyme 3.
3. PBS-2 mM EDTA.
4. 12 × 75 mm polypropylene Falcon tubes.
5. Antibodies to the protein of interest CD133 and matched isotype control.
6. 7AAD viability dye.

2.4 Intracranial Injections

1. Dulbecco's phosphate-buffered saline (PBS).
2. Rodent gas anesthesia instrument equipped with a vaporizer, isoflurane, oxygen tank, charcoal scavenger filters, induction chambers, and nose cones.
3. 56.8 % Petrolatum, 42.5 % mineral oil, Refresh Lacri-Lube.
4. Surgical povidone detergent.

5. Povidone-iodine surgical scrub.
6. Size 24 disposable safety scalpel.
7. 25 μ L 1702 RN Neuros Hamilton Syringe.
8. 17 mm Vicryl-coated absorbable suture; violet, braided.
9. 3 M Vetbond™ Tissue adhesive.
10. 3 μ g/mL buprenorphine/PBS.
11. Sterile 0.9 % sodium chloride for injection/injectable USP.

2.5 Mouse Brain Harvest and Immunohistochemistry Components

1. 2.5 % 2,2,2-Tribromoethanol (Avertin): Dissolve 5 g of Avertin powder in 5 mL of tert-amyl alcohol. Stir vigorously until dissolved. Heating in 50 °C water bath will help. Add 2.5 mL of Avertin-tert-amyl alcohol solution to 97.5 mL of pre-warmed PBS. Filter through 0.22 μ m filter and aliquot into glass vacutainer tubes, 5 mL per tube. Wrap the tubes in foil and store at 4 °C.
2. 1-in. sharp-ended scissors, 3-in. sharp-ended scissors, 3-in. blunt-ended scissors.
3. 6-in. sharp-ended forceps, 8-in. blunt-ended forceps.
4. 1 cc syringe and 1/2 cc insulin syringe per mouse.
5. 21 G needles to attach to saline and formalin lines.
6. 1,000 USP units/mL heparin sodium injection.
7. Saline solution, 0.9 % sodium chloride 1,000 mL bag.
8. 10 % formalin.
9. 1.0 mm coronal mouse brain slicer matrix and two razor blades.
10. 50 and 70 % ethanol.
11. 1 tissue-embedding cassette per mouse brain.

3 Methods

3.1 Tumor Processing

1. Obtain brain tumor sample and place in a sterile petri dish. Rinse with an adequate amount of artificial cerebral spinal fluid (aCSF) to remove red blood cells (RBCs).
2. Using sterile surgical scissors, disaggregate tumor into a “slurry” of homogenous consistency (*see Note 1*).
3. Transfer the dissociated cells into a 50 mL Falcon tube containing 15 mL aCSF and 200 μ L Liberase. Incubate at 37 °C for 15 min on an incubator-shaker to facilitate complete cell dissociation (*see Note 2*).
4. Following the 15-min incubation, filter tissue lysate through a 70 μ m nylon mesh strainer into a 50 mL Falcon tube to remove undigested tissue.

5. Centrifuge filtrate at $300\times g$ for 5 min.
6. Remove supernatant, resuspend cell pellet in 1 mL of PBS, and add an appropriate amount of ammonium chloride solution to mediate lysis of red blood cells (usually 4–12 mL). Incubate at room temperature for 5 min (*see Note 3*).
7. After 5 min, dilute RBC lysis buffer with 10 mL PBS and centrifuge cells at $300\times g$ for 5 min.
8. Remove supernatant and evaluate the cell pellet as this will determine the size of the ultralow attachment culture dish required for culture.
9. Resuspend the cell pellet in 1 mL of complete neural stem cell (NSC) media, transfer to either a 60 mm or a 100 mm ultralow culture petri dish, and top up to 4 mL or 10 mL, respectively (*see Note 4*).

3.2 Primary Cell Culture

Typically, cells may be cultured for days to weeks in NSC complete media. We have found that minimal manipulation and disturbance of primary cultures produce the most success. High-density, low-passage cultures may lead to a signaling environment more similar to the original tumor microenvironment, facilitating more robust growth and viability. Rather than regularly passaging cells, we recommend 1–2 mL media top-up when media becomes an orange color to maintain environmental consistency. Due to significant inter-tumor heterogeneity, each patient tumor behaves differently in culture necessitating careful observation of each sample. Experimentation, rather than protocol, has proven to be the most useful approach to the culture of these cells (*see Note 5*).

3.3 Sorting BTICs by Flow Cytometry

Flow cytometry sorting requires samples to be in single-cell suspension. Antibodies can then be used to label surface markers (proteins) of interest.

1. Dissociate neurospheres and cell aggregates into single-cell suspension using Liberase/Blendzyme. Transfer the entire culture into a 15 mL Falcon tube, add 10 mL PBS, and pellet by centrifugation at $300\times g$ for 5 min.
2. After centrifugation, carefully remove supernatant and resuspend pellet in 1 mL of sterile PBS. Add 10 μ L of Liberase and incubate for 3–8 min at 37 °C (*see Note 6*).
3. Add 10 mL of sterile PBS and centrifuge at $300\times g$ for 5 min.
4. Remove supernatant and resuspend the cell pellet in 500 μ L–1 mL of sterile PBS + 2 mM EDTA. Assess cell number and viability by trypan blue and/or a Countess® Automated Cell Counter (*see Note 7*).
5. Set aside a small aliquot (50–100 μ L) of the cell suspension which will serve as a negative control, either autofluorescent

or, if available, isotype. Specific antibodies are added to the rest of the cell suspension in amounts predetermined by titration.

6. The cell suspensions are incubated on ice for 30 min.
7. Wash cells once in PBS-EDTA by centrifugation at $300\times g$ for 4 min. Carefully remove supernatant.
8. Resuspend pellets to desired concentration in PBS+2 mM EDTA.
9. Add appropriate amount of chosen viability dye to each tube and incubate tubes on ice for at least 15 min.

3.4 Flow Cytometry Acquisition and Sorting

The specifics of acquisition, analysis, and sorting are instrument dependent. Basically, representative negative samples are run and instrument settings established for forward (size) and side (granularity) scatter. This scatter pattern allows the end user to view all cells in the sample, including debris. A region is usually drawn around the cells of interest. Using the negative control, instrument settings are then established for all fluorescence detectors required to position negative cells within the first decade of a fluorescence intensity plot. Any fluorescence beyond this level will be considered positive. When more than one dye or fluorochrome is used, single-stained controls are run to establish color compensation values. Viability dyes (such as 7-AAD) are used and a second region is drawn to exclude dead cells. Both of these regions are applied to plots depicting fluorescence associated with antibodies. Once the populations of interest are identified, additional regions are placed around them and then all regions involved are used to determine sort criteria (Fig. 1).

3.5 Intracranial Injections

To achieve a human-mouse xenograft model, immunodeficient mice are used for optimal engraftment of human cells. Due to the susceptibility of these mice to infection, all procedures must be performed in a designated clean room and within a BSL-2 hood.

1. Prepare required number of cells for injection. Resuspend in PBS so that the required number of cells to be injected is in 10 μ L. Keep cell solution on ice.
2. Set up the rodent anesthesia instrument as required.
3. Weigh mouse and place it in the induction chamber of the rodent gas anesthesia instrument (*see Note 8*).
4. Once the mouse is unresponsive to a foot pinch, place the mouse on a platform and apply Refresh Lacri-Lube to the eyes in order to prevent corneal drying.
5. Ensure that the nose of the mouse is properly inserted into the nose cone attached to the maintenance tube of the rodent anesthesia instrument, and proceed to gently secure the mouse to a stereotactic frame (*see Note 9*).

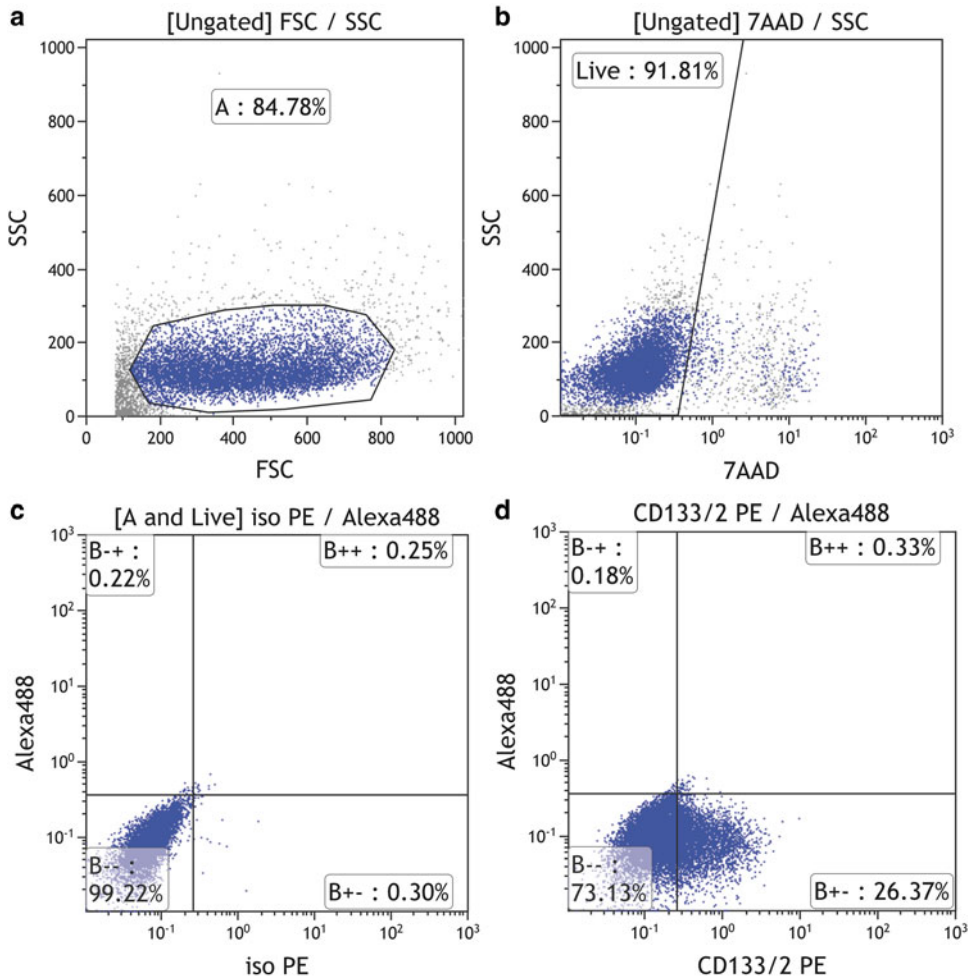


Fig. 1 Flow cytometric analysis of BTIC populations. (a) Forward (FSC) versus side scatter (SSC) properties elucidate an image of all cells, including debris. FSC is proportional to cell-surface area or size and SSC is proportional to cell granularity or internal complexity. (b) Cells stained with the viability dye 7-AAD are eliminated from analysis. (c) The position of the statistical quadrants is determined using appropriate isotype controls. (d) Glioblastoma BTICs are stained with surface markers CD133-PE and analyzed

6. Using a cotton swab, or another instrument of choice, clean the top of the mouse head with surgical detergent, followed by water and then a povidone-iodine surgical scrub.
7. Using a scalpel, make a sagittal cut down the midline of the mouse's head. This cut should run from between the eyes to the point that is equidistant from both mouse ears. Spread the skin as wide as possible.
8. Remove the periosteum from the skull by scraping it with a scalpel.
9. Locate the sagittal and coronal sutures, and identify the point located 2 mm behind the coronal suture and 3 mm to the right

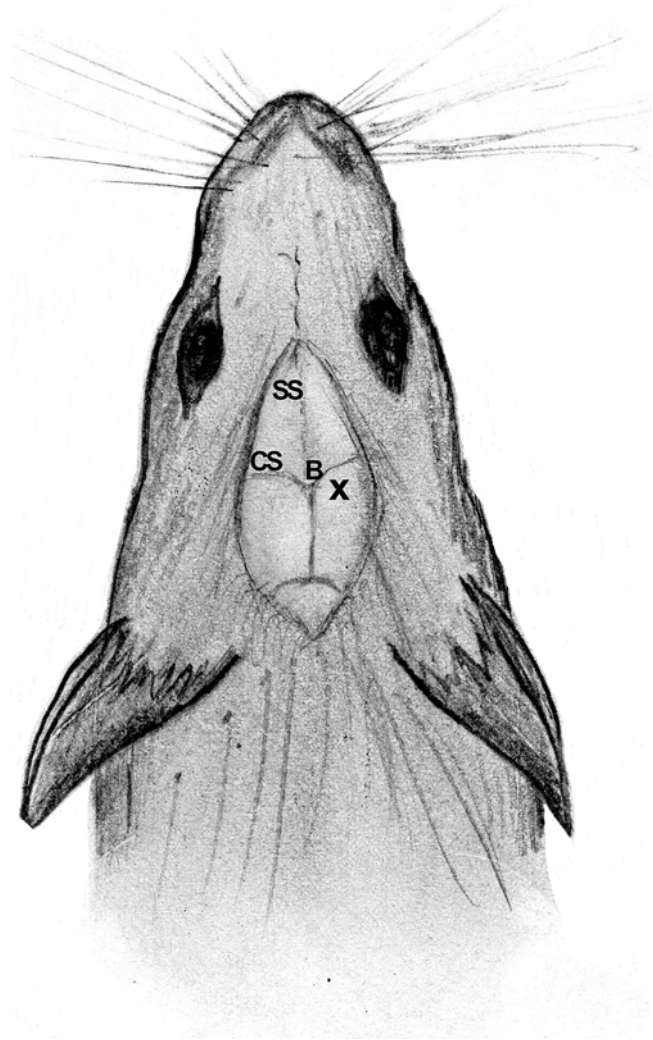


Fig. 2 Representative anatomical landmarks of mouse skull. The main anatomical landmarks are *highlighted*: bregma (B), coronal suture (CS), and sagittal suture (SS). The approximate location of burr hole and site of injection is marked by X

of the sagittal suture (Fig. 2). Drill a burr hole by tapping the drill bit lightly against the skull. Drill until a reddish area is visible, or until a Hamilton syringe can penetrate through the remaining skull (*see Note 10*).

10. Uptake 10 μ L of cell solution into a Hamilton syringe.
11. Insert the Hamilton syringe 3 mm into the burr hole at either a 90° or a 60° angle from the horizontal.
12. In one smooth, uninterrupted motion, slowly inject the 10 μ L of cell suspension into the murine frontal lobe. Tap the end of the syringe three times before removing to ensure that the

drop remains in the brain and is not displaced with the removal of the Hamilton syringe.

13. Remove any spilled blood with gauze, and suture the wound with 2–3 stitches using a simple interrupted technique. Add two extra throws after the initial knot has been tied and cut off any excess suture thread.
14. Apply a small amount of tissue adhesive to the sutured cut.
15. Mark mice as necessary (i.e., tail mark, ear notch).
16. Inject 1 mL of sterile 0.9 % sodium chloride subcutaneously by inserting the syringe into the scruff of the neck or the gluteal region of the mouse.
17. Inject 0.5 mL of buprenorphine (Temgesic) subcutaneously by inserting the syringe into the scruff of the neck or the gluteal region of the mouse.
18. Place mouse in a cage on a piece of gauze, and position the cage near a heat source (heat lamp or heat pad) until the mouse awakens (*see Note 11*).
19. On the first day following surgery, inject the mouse with 1 mL of sterile 0.9 % sodium chloride and 0.5 mL of buprenorphine (Temgesic) subcutaneously.
20. Observe mouse for desired length of time or until experimental endpoint.

3.6 Preparation of Mouse Brain for Harvesting and Immunohistochemistry

Ensure that all dissecting tools including forceps and scissors are sterile to prevent contamination of mouse brain tissue. All procedures must be performed in a BSL-2 hood unless otherwise noted.

3.6.1 Anesthetizing the Animal

1. Draw up 18 μ L of 2.5 % Avertin per gram of mouse weight into a 1 cc syringe.
2. Retrieve and securely restrain the animal, tilt the head back, and administer the Avertin through an intraperitoneal injection. Return the animal to its cage.
3. Ensure that the mouse is properly sedated by pinching the toes to ensure that there is no response to painful stimulus.

3.6.2 Mouse Brain Harvest

1. Perform cervical dislocation as instructed by your animal facility.
2. Decapitate the mouse at the site of dislocation. Remove the skin from base of the head to the nose exposing the scalp.
3. Using small sharp-ended scissors, pierce the skull at the base of the head and carefully cut till the ridge between the eyes.

4. Using sharp-ended forceps, remove the skull by carefully pulling it away from the brain until all of the brain is exposed (*see* **Note 12**).
5. Carefully lift the brain out of the skull in a scooping manner (*see* **Note 13**) and place it in ~15 mL of sterile PBS.
6. Culture the mouse brain as mentioned previously in Subheading 3.1.

3.6.3 Mouse Brain Fixation

1. Place the anesthetized mouse on an inclined surface with abdomen facing up. Spread the paws as wide as possible and secure with tape.
2. Using blunt-ended forceps, grab skin at the level of the diaphragm and cut with blunt-ended scissors to expose the liver. Cut laterally and then up through the ribs. Be sure to draw scissors away from organs to avoid unnecessary damage to the circulation. Continue cutting through the ribs until the heart is easy to access (*see* **Note 14**).
3. Inject 20 μ L of heparin using $\frac{1}{2}$ cc syringe into the left ventricle. Wait for 10–12 heartbeats.
4. Hold the heart gently with forceps. Turn on the saline flow (~2 mL/min) and place the needle in the left ventricle. Insert gently no more than $\frac{1}{4}$ inch. Then immediately cut the right atrium. If the cut is sufficient you should see a flush of blood in the body cavity as the pressure from the saline is relieved from the heart.
5. Continue in saline until the liver changes color to light coffee brown.
6. Remove the saline needle and replace it with 10 % formalin flow (~2 mL/min). Make sure that the needle enters through the same hole as the saline (*see* **Note 15**).
7. Decapitate and remove the mouse brain as detailed previously in Subheading 3.5, **step 1**, and place it in ~15 mL of 10 % formalin.
8. Store in -4°C fridge for at least 48 h of fixation until ready for immunohistochemistry.
9. Place the brain in a 1 mm coronal mouse brain slicer matrix. Using sharp razor blades, slice the brain at every other channel to get ~2 mm thick slices. This procedure does not need to be performed in a BSL-2 hood.
10. Place mouse brain slices in cassettes and dehydrate in 50 % ethanol for 5 min followed by 70 % ethanol for at least 24 h. This prepares the brain slices for paraffin embedding. IHC may now be performed as desired (Fig. 3).

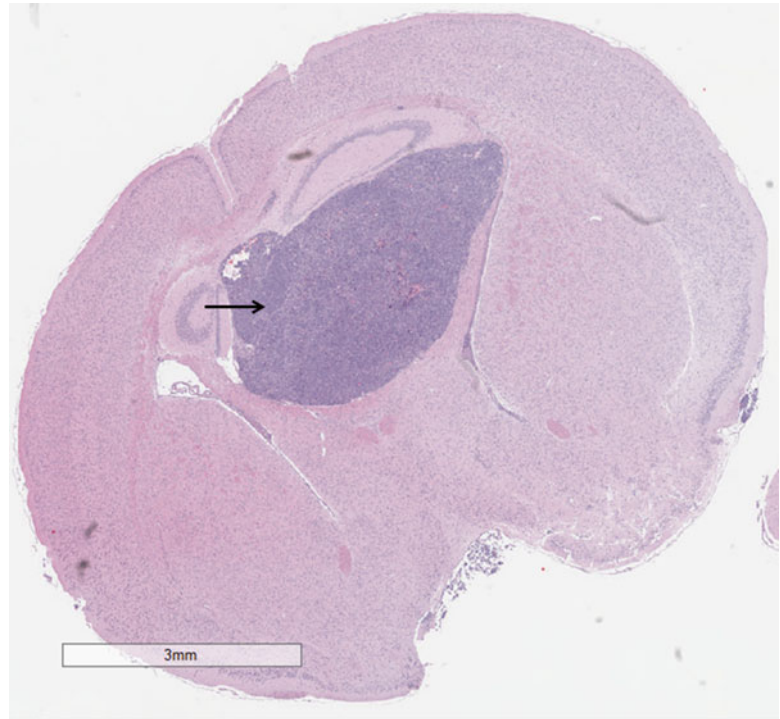


Fig. 3 Representative IHC-stained mouse xenograft. Hematoxylin and eosin (H&E) staining of coronal section of mouse brain from Med8A (medulloblastoma cell line) injected mice reveal a large tumor mass (*arrow*)

4 Notes

1. When tumor has been homogenized, take care not to expose cells to air for extended periods of time to prevent unnecessary dryness and cell death.
2. Seal the 50 mL Falcon tube with parafilm to prevent leakage during the 15-min rocking incubation.
3. Vascularity and RBC content of tumors are highly variable, as is size, so the exact amount of RBC lysis buffer is at the user's discretion.
4. We have found that low cell density of tumor cells is less conducive to healthy growth and survival of tumor cells. Therefore, in most situations, a 60 mm culture dish with 5 mL NSC complete media is recommended.
5. Note that in the described conditions, cells will be non-adherent. The development and growth of spheroid bodies (termed neurospheres) is common. Many neurospheres are clonal colonies derived from a single BTIC or progenitor cell, and are enriched for stemlike populations.

6. Cells will tend to settle to the bottom of the Falcon tube during dissociation. It is recommended to use a p1000 pipette to resuspend cell suspension every 3 min until complete dissociation is achieved. Note that incomplete cell dissociation results in clumps of cells and will negatively impact the efficiency of flow cytometry, while excessive exposure to Liberase is toxic.
7. If using a countess system, this step presents an ideal opportunity to examine the counted cells for complete dissociation. If clumps of 2–3 cells are still visible, dissociation step will have to be extended to achieve single suspension.
8. The vaporizer of the isoflurane anesthetic machine can be set between 3 and 5 % when the mouse is in the induction chamber.
9. The mouse must be kept under maintenance anesthesia (vaporizer dial at 2.5 %) to ensure that the mouse does not awake during the surgery.
10. We have found that a burr hole is usually achieved after approximately ten gentle taps of the drill bit against the mouse skull.
11. While placing the mouse on gauze, make sure that the mouse's air passages are unobstructed to allow for proper respiration. This allows for good recovery post-surgery.
12. The skull can be removed in small pieces to prevent damage to the brain.
13. Be careful around the olfactory bulbs as they are easy to damage. Make sure that the entire skull is removed around the olfactory bulbs to prevent damage while removing the brain.
14. Free the heart by tearing any connective tissue with forceps and not scissors.
15. A good indication of how well the animal is being fixed is to test tail flexibility, which should be stiff.

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Growth of Bone Marrow and Skeletal Muscle Side Population Stem Cells in Suspension Culture

Christina A. Pacak and Douglas B. Cowan

Abstract

The ability to efficiently isolate and expand various stem cell populations in vitro is crucial for successful translation of cell-based therapies to the clinical setting. One such heterogeneous population that possesses a remarkable potential for the development of cell-based treatments for a variety of degenerative diseases and disorders is called the Side Population (SP). For many years, investigators have isolated these primitive cells based upon their ability to efflux the fluorophore Hoechst 33342. This attribute enabled separation of SP cells derived from multiple tissue sources from other endogenous cell populations using fluorescence-activated cell sorting (FACS). While all tissue-specific SP fractions appear to contain cells with multi-potent stem cell activity, the therapeutic utility of these cells has yet to be fully realized because of the scarcity of this fraction in vivo. In view of that, we developed a method to expand adult murine bone marrow and skeletal muscle-derived SP cells in vitro. Here, we describe a spinner-flask culture system that supports the growth of SP cells in suspension when they are combined with feeder cells cultured on spherical microcarriers. In this way, their distinguishing biological characteristics can be maintained, attachment-stimulated differentiation is avoided, and therapeutically relevant quantities of SP cells are generated. Modification of the described procedure may permit expansion of the SP from other relevant tissue sources and our method is amenable to establishing compliance with current good manufacturing practices.

Key words Side population cells, SP cells, Adult stem cells, Cell expansion, Microcarrier, Skeletal muscle, Bone marrow, Spinner flasks, Suspension culture

1 Introduction

There remains widespread interest in employing Side Population (SP) stem cells isolated from various tissue sources, including skeletal muscle and bone marrow, for cell-based therapies directed at regenerating or repairing damaged tissue. However, there are significant obstacles to overcome before any such treatment would be considered clinically relevant [1–3]. The inability to dependably grow ex vivo cultures of this population continues to represent a fundamental impediment to translating SP cell-based therapeutics to the clinic. A clearly defined and well-controlled means to consistently expand SP cells in culture would enable sufficient cell yields

to be generated for further experimental characterization of this fraction and evaluation of these cells as an autologous, regenerative stem cell source in humans.

Thus far, there have been few attempts to culture primary SP cells specifically for *in vitro* expansion. In general, SP cell cultures produce small, uniform, non-adherent, rounded cells that demonstrate limited ability to proliferate [4–7]. Previously published reports using cultured SP cells utilized various matrices (e.g., methylcellulose or collagen) in combination with supplements to study the differentiation potential of this fraction [8–14]. Although these approaches did promote some proliferation of differentiated hematopoietic lineages, they were never intended to yield sufficient numbers of undifferentiated SP cells for repair of tissues of mesenchymal lineage like the heart or skeletal muscle.

Here, we describe a three-dimensional (3D) coculture bioreactor to grow SP cells from two commonly used tissue sources; namely, skeletal muscle and bone marrow [15]. We reasoned that successful expansion of undifferentiated SP cells would require contributions from other cells normally associated with their *in vivo* microenvironment or niche. For this reason, we decided to maintain the SP in coculture with “feeder” cells that would presumably provide growth factors and, possibly, other conditions necessary for long-term, *in vitro* cell survival and proliferation. At the same time, we sought to provide a simple means to separate expanded SP cells from feeder cells. To achieve this, we employed a microcarrier-based suspension culture system. Using this approach, feeder cells were grown as a single layer on the surface of small spheres and maintained in suspension using magnetic impeller-driven spinner flasks placed on a stir plate in a tissue culture incubator [15–17]. Following an equilibration period, SP cells were added to these bioreactor flasks to determine their growth and differentiation status over the course of several passages.

The culture system was designed to allow for effortless separation of feeder cells from suspended SP cells. Conceptually, this separation would be performed by allowing the relatively heavy microcarriers (with feeder cells attached to them) to sink to the bottom of the spinner flasks by simply removing them from the magnetic stir plate. Suspended cells and settled microcarriers would then be collected separately. In practice, we found SP cells often aggregate around the feeder cell-covered microcarriers and they need to be harvested using mild enzymatic and mechanical dissociation techniques [15]. On the other hand, de-cellularized microcarriers can be readily separated from cocultures by filtration. Despite the inconvenience of working with mixed cell populations, we found a large number of expanded SP cells, whether derived from bone marrow or skeletal muscle, retain the ability to efflux Hoechst dye and possess a protein expression profile consistent with freshly isolated, unfractionated SP cells. While some SP cells

grown in culture do undergo lineage commitment and differentiation, they can be easily distinguished from undifferentiated cells using flow cytometry and protein expression analyses.

In summary, we have developed a method to grow SP stem cells using a microcarrier-based coculture bioreactor system. This method allows for expansion of this rare cell fraction through multiple passages and collection of bone marrow and skeletal muscle-derived SP cells that retain attributes of freshly isolated cells. We believe this culture system could be modified to support the growth of SP cells from other tissue sources, such as the heart, by simply changing the type of feeder cells to be attached to the microcarriers [18]. In addition, the method described here should stimulate further study of this cell fraction with the goal of streamlining the process of isolating and purifying these cells. Ultimately, we anticipate that this method will establish the therapeutic potential of the SP in regenerating or repairing damaged tissue.

2 Materials

2.1 *Disposables*

1. Mice (C57Bl/6 or C57BL/6-Tg[ACTB-EGFP]1Osb/J strains) (Jackson Laboratory).
2. 70 % Ethanol (EtOH).
3. Hanks Buffered Salt Solution (HBSS).
4. Razor blades.
5. Muscle Digestion Buffer: 100 mL Dispase 2, 1 g Collagenase 2, 0.037 g CaCl_2 passed through a 0.2 μm syringe filter and stored at -20°C .
6. 40 μm Cell Strainer.
7. 100 μm Cell Strainer.
8. $1\times$ Ca^{2+} and Mg^{2+} - free Phosphate Buffered Saline.
9. $1\times$ PBS containing 0.5 % bovine serum albumin ($1\times$ PBS + 0.5 % BSA).
10. Red blood cell lysis buffer: 0.2 % Tris-HCl (pH 7.5), 0.747 % NH_4Cl (store at 4°C).
11. Hoechst 33342.
12. Verapamil.
13. Propidium iodide.
14. C2C12 myoblast cell line.
15. 150 mm tissue culture plates.
16. C2C12 expansion medium: High-glucose Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX with sodium pyruvate and phenol red, 20 % fetal bovine serum (FBS), 1 % penicillin–streptomycin, 1 % Fungizone.

17. 0.05 % trypsin–EDTA.
18. 15 and 50 mL CELLSTAR conical tubes.
19. Cytodex 1 microcarriers.
20. Petri dishes.
21. Sigmacote.
22. Basic fibroblast growth factor (bFGF).
23. Mouse bone marrow or skeletal muscle.
24. 1 % Trypsin.
25. Disposable serological pipettes (1, 5, 10, and 25 mL sizes).
26. Multi-wipes laboratory wipes.
27. 23 G 1" needle and 3 cc syringe.

2.2 Equipment

1. Dissection tools (scissors, scalpels, forceps).
2. Centrifuge.
3. Humidified cell culture incubator.
4. Fluorescence Activated Cell Sorter.
5. 100 mL borosilicate glass spinner flasks with an internal overhead assembly or adjustable hanging bar with curved polytetrafluoroethylene paddle.
6. 250 mL borosilicate glass spinner flasks with an internal overhead assembly or adjustable hanging bar with curved polytetrafluoroethylene paddle.
7. Bellenium 5-position magnetic stirrer or MultiMagStir Genie.
8. Enviro-Genie incubator.
9. Autoclave.
10. SterilGARD III Advance° Laminar Flow Hood.
11. Portable Pipette-Aid.

3 Methods

3.1 Preparation of Microcarrier Suspension Cultures

1. Culture myoblast feeder cells on 150 mm plates in C2C12 expansion medium (Fig. 1), (*see Note 1*).
2. Hydrate and sterilize 1×10^6 Cytodex 1 microcarriers using purified water, 70 % ethanol, PBS and C2C12 expansion medium according to the manufacturer's directions.
3. Coat two 100 mL borosilicate glass spinner flasks with Sigmacote and sterilize for 20 min in the autoclave on the dry cycle.
4. Remove the medium from approximately 1×10^8 C2C12 cells by aspiration and rinse the cells twice with 37 °C $1 \times$ PBS (*see Note 2*).

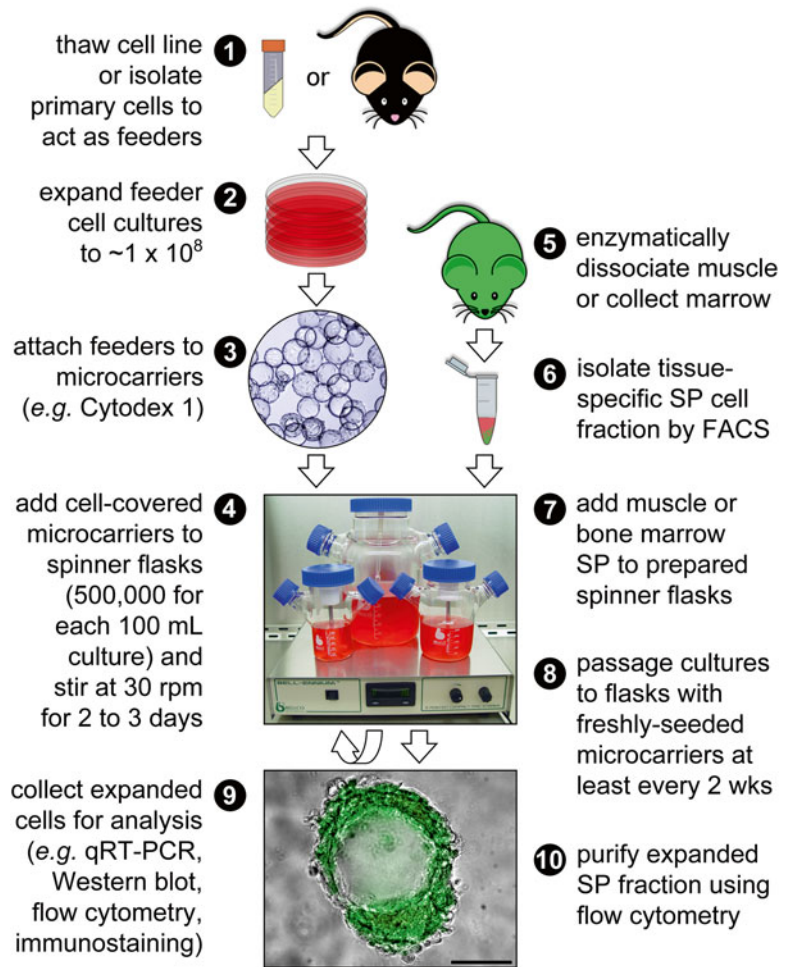


Fig. 1 Schematic overview of the method to expand SP stem cells in suspension. The C2C12 myoblast cell line was used as the feeder cell layer on microcarriers; however, other adherent cell lines or appropriate primary cells could be used. Here, we employed SP cells isolated from mice expressing enhanced green fluorescent protein (EGFP) to allow the expanding SP population to be distinguished from the feeder cells. The procedural order is indicated by numbers and the scale bar = 100 μm

5. Detach cells from each plate using 3 mL of pre-warmed 0.05 % trypsin–EDTA at 37 °C for 2–5 min.
6. Add 5 mL expansion medium to each plate to inactivate the trypsin and help detach cells from the culture plate surface.
7. Collect the detached cells in 50 mL conical tubes and centrifuge at 600 $\times g$.
8. Remove the supernatant and resuspend cell pellets in a total 30 mL 37 °C C2C12 expansion medium and combine with the prepared microcarriers.

9. Divide the entire preparation between 6 and 8 100 mm petri dishes and place in a humidified culture incubator with 5 % CO₂. Agitate the dishes every 15 min for 2 h by gently shaking the dishes back and forth (*see Note 3*).
10. Assemble two sterilized, Sigmacote-treated 100 mL glass spinner flasks in the culture hood, if necessary.
11. Divide the feeder cell-covered microcarriers equally between the two spinner flasks (i.e., 500,000 in each flask) and fill to 100 mL with 37 °C C2C12 expansion medium.
12. Loosen the side-arm caps half way to allow for gas transfer in the culture incubator.
13. Stir the flask contents at 30 rpm using a magnetic stirrer placed in a humidified tissue culture incubator (*see Note 4*).

3.2 Isolation of Skeletal Muscle Cells

1. Euthanize a mouse using CO₂-asphyxiation followed by cervical dislocation. Pin down limbs on a silicone dissection pad and soak the fur with 70 % EtOH.
2. Extract the major hind-limb muscles (quadriceps, gastrone-mius, tibialis anterior), forelimb muscles (triceps), as well as the paraspinal muscles and place these in a 50 mL conical tube containing 25 mL HBSS (*see Note 5*).
3. Transfer all skeletal muscle to a 100 mm petri dish with approximately 2 mL HBSS and using forceps and scalpel, remove as much fat and connective tissue as possible (*see Note 6*).
4. Transfer the muscle pieces to a clean petri dish with approximately 2 mL HBSS.
5. Using two razor blades finely mince the muscle into slurry using sterile gloves.
6. Add 10 mL of 37 °C muscle digestion buffer.
7. Place muscle slurry into a 50 mL conical tube with warm diges-tion buffer.
8. Digest for approximately 45 min or until tissue fragments are no longer apparent using an Enviro-Genie on the 15:30 rocking setting at 37 °C.
9. Throughout the digest triturate every 5 min by drawing the liquid up and down with a disposable serological pipette (*see Note 7*).
10. Place a 100 µm Cell Strainer over a sterile 50 mL conical tube to filter out connective tissue and undigested fragments from the digestion solution.
11. Rinse the Cell Strainer with 1× PBS+0.5 % BSA to collect residual cells trapped in the nylon mesh.

3.3 Isolation of Bone Marrow Cells

1. Dissect skeletal muscle and other tissue from the region surrounding the femur and tibia in situ.
2. Remove these bones from the body by cutting as close to the pelvis as possible while avoiding fragmenting the bones.
3. Carefully and thoroughly clean the bones using a razor blade and laboratory wipes (*see Note 8*).
4. Cut at the knee joint to separate the femur and tibia.
5. Use a 23 G needle and 3 cc syringe to inject 1× PBS+0.5 % BSA into the marrow cavity of each bone and force the contents out the other end into a clean 15 mL conical tube.
6. Dilute the bone marrow/PBS+0.5 % BSA mixture (usually ~3 mL) 1:7 with red blood cell lysis buffer (usually ~21 mL).
7. Strain the solution through a 100 µm Cell Strainer collecting the wash-through in a 50 mL conical tube.
8. Rinse the Cell Strainer with 1× PBS+0.5 % BSA to collect residual cells trapped in the nylon mesh.

3.4 Preparation of Isolated Skeletal Muscle or Bone Marrow Cells for Flow Cytometry

1. Filter the dissociated skeletal muscle and bone marrow cell suspensions through a 40 µm Cell Strainer and collect flow-through using a 50 mL conical tube.
2. Rinse the Cell Strainer with 1× PBS+0.5 % BSA to collect residual cells trapped in the nylon mesh.
3. Centrifuge ($600\times g$) at 4 °C for 10 min and resuspend to 1×10^6 cells/mL in 1× PBS+0.5 % BSA.
4. Add 100 mM verapamil to an aliquot of each cell type to provide a negative control for SP cell gating.
5. Add Hoechst 33342 at a concentration of 5 µg/mL for bone marrow cells and 12.5 µg/mL for skeletal muscle cells (i.e., both the primary cell suspensions and the control aliquots containing verapamil) and stain in the dark for 90 min at 37 °C using a water bath.
6. Wash all stained cells with 5 volumes of PBS+0.5 % BSA.
7. Prior to flow cytometric analysis and sorting, resuspend the cells in PBS+0.5 % BSA containing 2 µg/mL propidium iodide for 5 min and store on ice (*see Note 9*).

3.5 Fluorescence Activated Cell Sorting (FACS) of SP Cells

1. Perform cell sorting using a FACS machine capable of generating an excitation wavelength of 365 nm.
2. Measure fluorescence emission using a 400 nm long-pass filter for Hoechst 33342 and a 600 nm long-pass filter for propidium iodide.
3. Set the sort head frequency at 26,000 Hz using the “normal sort” mode.

4. Set the sheath pressure to 11–12 psi.
5. Following cytometric analysis of a portion of each sample, set the gate to select for live cells (the fraction emits the least red signal) with the lowest Hoechst dye concentrations (the fraction emits the least blue signal) (*see* **Note 10**).

3.6 Inoculation and Maintenance of Suspension Cultures

1. 2–3 days after setting up the microcarrier spinner flasks (steps described in Subheading 3.1), supplement the culture medium with 5 ng/L bFGF and inoculate each flask with freshly isolated SP cells (*see* **Note 11**). This culture is referred to as passage 0 (P0).
2. To replenish medium, remove the flasks from the stir plate and allow the microcarriers and SP cells to settle to the bottom of the flasks (5–10 min) (*see* **Note 12**).
3. Slowly remove 50–70 % of the medium from the top portion of the flask and replace it with fresh pre-warmed medium containing 5 ng/L bFGF (*see* **Note 13**).

3.7 Passaging of Suspension Cultures

1. No more than 2 weeks after SP cell inoculation, collect the contents of each flask by allowing cells and microcarriers to settle on the bottom of the flask (*see* **Note 14**).
2. Remove most of the medium and transfer the cell and microcarrier aggregates to 50 mL conical tubes.
3. Allow the contents to settle and remove as much medium as possible.
4. Rinse the cell and microcarrier aggregates twice with 1× PBS.
5. Incubate the aggregates with about 5 mL 1 % trypsin and agitate using the Enviro-Genie incubator at 37 °C with a 5:10 rocking setting for 10 min.
6. Stop the trypsin reaction by adding 10 mL FBS.
7. Remove the microcarriers from the rest of the dispersed cell suspension by passing the mixture through a 100 µM Cell Strainer.
8. Rinse the Cell Strainer with 1× PBS+0.5 % BSA to collect residual cells trapped in the nylon mesh.
9. Centrifuge the filtered solution at 600×g for 10 min at room temperature.
10. Remove supernatant and resuspend cell pellets in 25 mL each of expansion medium containing bFGF.
11. Add these resuspensions to 250 mL spinner flasks each containing 1×10^6 microcarriers prepared and seeded with C2C12 cells as described above (*see* **Notes 15 and 16**). These cultures are referred to as passage 1 (P1).
12. In the next passage, cells are split into 2×250 mL spinner flasks (P2) and then 4×250 mL spinner flasks for each culture (P3).

4 Notes

1. We used the C2C12 cell line to develop this method; however, Main Population (MP) cells from bone marrow or skeletal muscle could be used for this purpose. Alternatively, if SP cells were isolated from a different tissue source, an appropriate tissue-specific feeder cell would be desirable.
2. Usually four to six 50–70 % confluent 150 mm tissue culture dishes provide 1×10^8 C2C12 cells. C2C12 cells should be passaged at no more than 70 % confluence to prevent fusion and differentiation of these cells into multinucleated myotubes.
3. Petri dishes should be used rather than tissue culture plates as feeder cells will not attach to this plastic. This forces the feeders to adhere to the microcarrier surface.
4. The stir bar and paddle assembly should rotate at a steady rate and not in an erratic manner. A stable, continuous mixing of the culture minimizes the chance of cells detaching from the microcarrier surface as a result of intermittent exposure to shear forces.
5. Keep muscles wet with $1 \times$ PBS throughout muscle isolation procedure to maintain cell viability.
6. This step and those immediately following it should be performed in a laminar flow hood.
7. Avoid bubbling the liquid during trituration and use progressively smaller volume pipettes as the decreasing entrance hole diameter will help break the tissue down.
8. Meticulous removal of all cells and tissue from the outside of the bones reduces the chance of non-marrow contaminants and permits visualization of the marrow.
9. It is very important to keep the cells on ice and move quickly to the FACS machine to acquire consistent data as well as viable cells to culture following the sort.
10. What has been described here is, by definition, the side population or SP. This is a greatly abbreviated version of the steps required to identify and purify this stem cell-containing population. There are numerous publications describing these steps in greater detail [5, 19, 20].
11. Generally, from a single mouse there will be 10,000–15,000 muscle SP cells and 20,000–50,000 bone marrow SP cells.
12. It is absolutely crucial that the medium in SP cell suspension/expansion flasks is replenished every 2–3 days.
13. Small aliquots of the culture (5 mL) can be collected from the side arms of the suspension flasks as needed for enumeration and analysis (Fig. 1).

14. If cultures are allowed to grow longer than 15 days before passaging, the feeder cells will begin to detach from the micro-carriers, which negatively impacts SP expansion.
15. As the culture continues to expand the cells can continue to be passaged every 2 weeks by dividing the SP cell yield from each flask in $\frac{1}{2}$ and using each $\frac{1}{2}$ to seed a freshly prepared 250 mL spinner flask.
16. Increasing the spinner-flask size beyond the 250 mL volume (i.e., 1,000 mL) results in a collapse in the culture, likely due to insufficient gas exchange within the culture.

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Chapter 6

Isolation, Culture and Immunostaining of Skeletal Muscle Fibres to Study Myogenic Progression in Satellite Cells

Louise A. Moyle and Peter S. Zammit

Abstract

Satellite cells are the resident stem cells of skeletal muscle, located on the surface of a myofibre, beneath the surrounding basal lamina. Satellite cells are responsible for the homeostasis, hypertrophy and repair of skeletal muscle fibres, being activated to enter proliferation and generate myoblasts that either fuse to existing myofibres, or fuse together for de novo myofibre formation. Isolating muscle fibres allows the associated satellite cells to be obtained while remaining in their anatomical niche beneath the basal lamina, free of interstitial and vascular tissue. Myofibres can then be immunostained to examine gene expression in quiescent satellite cells, or cultured to activate satellite cells before immunostaining to investigate gene expression dynamics during myogenic progression and self-renewal. Here, we describe methods for the isolation, culture and immunostaining of muscle fibres for examining satellite cell biology.

Key words Satellite cell, Stem cell, Skeletal muscle, Muscle fibre, Myofibre, Culture, Immunostaining, Self-renewal

1 Introduction

Skeletal muscle constitutes ~30 % of body mass for women and ~38 % for men [1]. The main role of skeletal muscle is to produce force, which occurs within repeat units called sarcomeres, when myosin filaments slide along actin filaments in the presence of ATP. Skeletal muscles usually comprise bundles of muscle fibres arranged in parallel. Myofibres are formed when mononucleate muscle precursor cells fuse during development and growth, to produce a syncytium, with each myofibre often containing hundreds of myonuclei in mammals [2].

In most vertebrates, once muscle precursors fuse, they undergo terminal differentiation and cannot reenter the cell cycle. However, skeletal muscle has a remarkably good ability to regenerate following injury [3–5]. This is due to a population of resident stem cells called satellite cells, located beneath the basal lamina of a myofibre [6–8]. Due to the low turnover of skeletal muscle in healthy adults,

satellite cells are usually mitotically quiescent. However, in response to damage they rapidly activate and proliferate to generate myoblast progeny that undergo myogenic differentiation to repair or replace damaged myofibres [9, 10].

Other non-satellite cell populations have been shown to have myogenic capacity under certain circumstances [11–13]. However, in a series of elegant experiments it has been shown that if the satellite cell population is genetically ablated, skeletal muscle does not regenerate [14–17] (reviewed in ref. [18]). Hence, satellite cells are essential for skeletal muscle repair. It is therefore important to understand the behavior and regulation of satellite cells during quiescence, activation, proliferation, self-renewal, and differentiation in both health and disease.

1.1 Origins of the Methodology

Here, we describe a method for isolating individual myofibres with associated satellite cells from the *extensor digitorum longus* (EDL) muscle of the mouse using enzymatic digestion. The origins of this technique can be traced back to the 1970s. Intact myofibres from mouse *gastrocnemius* were isolated using guanidine-HCl on fixed tissue by Cardasis and Cooper to analyze nuclear content [19, 20]. In 1975, Bischoff and Konigsberg reported that physically peeling myofibres from either unfixed adult rat muscle [21] or juvenile quail muscle [22] allowed satellite cells to be cultured. Kopriva and Moss had reported that muscle fibres/fragments could be obtained from rat *tibialis anterior* using digestion with collagenase [23], and Bekoff and Betz combined collagenase digestion and trituration to isolate complete viable muscle fibres from rat *flexor digitorum brevis* [24], which could be maintained in culture [25]. Analysis of satellite cells associated with isolated myofibres was optimized and championed by Bischoff in a series of papers through the 1980s and 1990s examining their activation and proliferation using mainly rat *flexor digitorum brevis* muscles, beginning with his defining 1986 paper [26]. Yablonka-Reuveni and Rivera applied immunostaining for MyoD and MyHC to myofibres to describe myogenesis in the associated satellite cells [27]. Isolation and culture of single muscle fibres from the longer, more fragile mouse EDL, *soleus*, and *tibialis anterior* muscles was pioneered by Rosenblatt and Partridge [28]. They also showed that myofibres attach to Matrigel, and satellite cells would then migrate from under the basal lamina and onto the tissue culture dish [29]. Zammit, Heslop and colleagues subsequently optimized the techniques for immunostaining quiescent mouse satellite cells [30] and described the non-adherent culture, and immunostaining of activated, proliferating and differentiating satellite cell progeny on isolated myofibres [10].

In this chapter we describe the isolation of viable myofibres and their associated satellite cells from the murine EDL muscle. The mouse EDL has approximately 1,000 muscle fibres [31] containing a mean of four satellite cells on each [2] and a good preparation can easily liberate 100 s of viable myofibres. The methodology described

below for isolation of myofibres from EDL muscle is up dated from our previous version [32]. Myofibres can be isolated from many other muscles, including those of the head, so is also useful for comparing satellite cells from different developmental origins [33]. We then detail non-adherent culture of myofibres to allow the associated satellite cells to activate, proliferate, and enter differentiation or self-renewal, and their analysis by immunostaining. Finally, we describe how myofibres can be plated to obtain large quantities of satellite cell-derived myoblasts for measuring gene expression and examining the later stages of myogenic differentiation, such as myoblast fusion into large multinucleated myotubes.

2 Materials

2.1 Equipment

1. Water bath at 37 °C.
2. Dissection microscope.
3. Stereo dissection microscope with transmission illumination.
4. Tissue culture hood or lamina flow cabinet.
5. Tissue culture incubator (humidified, 37 °C, 5 % CO₂).
6. Bunsen burner.
7. Cork dissection board.
8. Dissection pins.
9. Fine forceps, two pairs.
10. Forceps with teeth, 1 pair.
11. Vanna microscissors.
12. Sterile disposable scalpels No. 11.
13. Diamond pen.
14. Rubber pipette bulbs, 1.5 ml.
15. Deep Petri dishes (50 mm × 20.3 mm).
16. Glass Pasteur pipettes (22 cm), sterile.
17. Plastic pipettes (5, 10, 25 ml volumes), sterile.
18. Syringes 5 and 20 ml, sterile.
19. 0.2 and 0.45 µM syringe filters.
20. Bijou tubes, 7 ml.
21. Tilt table.
22. Aluminium foil.

2.2 Additional Materials Required for Cell Culture and Fixation

1. Dulbecco's modified Eagle's medium (DMEM), high glucose, GlutaMAX™, Pyruvate.
2. Penicillin and streptomycin solution.
3. Collagenase from *Clostridium histolyticum*.

4. Ethanol solution, 70 %.
5. Bovine serum albumin (BSA).
6. Phosphate-buffered saline (PBS), sterile.
7. Horse serum (HS).
8. Chick embryo extract.
9. Crystal-clear microcentrifuge tubes, 2 ml.
10. Petri dishes, 35 mm × 11 mm.
11. Goat serum.
12. Carrageenan.
13. Primary antibodies.
14. Fluorescein-conjugated Alexa Fluor® secondary antibodies.
15. Triton X-100 Surfact-Amps Detergent Solution.
16. Tween-20.
17. 4 % Paraformaldehyde (PFA).
18. Parafilm.
19. Cover glasses 22 mm × 25 mm × 0.5 mm.
20. Glass slides.
21. Vectashield containing DAPI.
22. Liquid blocker super pap pen.
23. Foetal bovine serum (FBS).
24. Recombinant murine FGF basic.
25. Matrigel.
26. Non-coated Petri dishes 60 mm, sterile.
27. Trypsin-EDTA solution 0.25 %.
28. Nunc® Labtech 8-well chamber slides.

3 Methods

3.1 Preparation

Where possible, all steps are performed under sterile conditions in a tissue culture hood or lamina flow cabinet.

1. Prepare DMEM by adding penicillin and streptomycin solution to 1 % vol/vol.
2. Prepare a 5 % BSA solution in sterile PBS and heat inactivate at 60 °C for 60 min before filtering through a 0.45 µm syringe filter.
3. Rinse 3–4 50 mm × 20.3 mm deep Petri dishes per muscle with 5 % BSA/PBS solution to prevent fibres from adhering to the dish. Remove excess BSA solution and add 8 ml of DMEM per

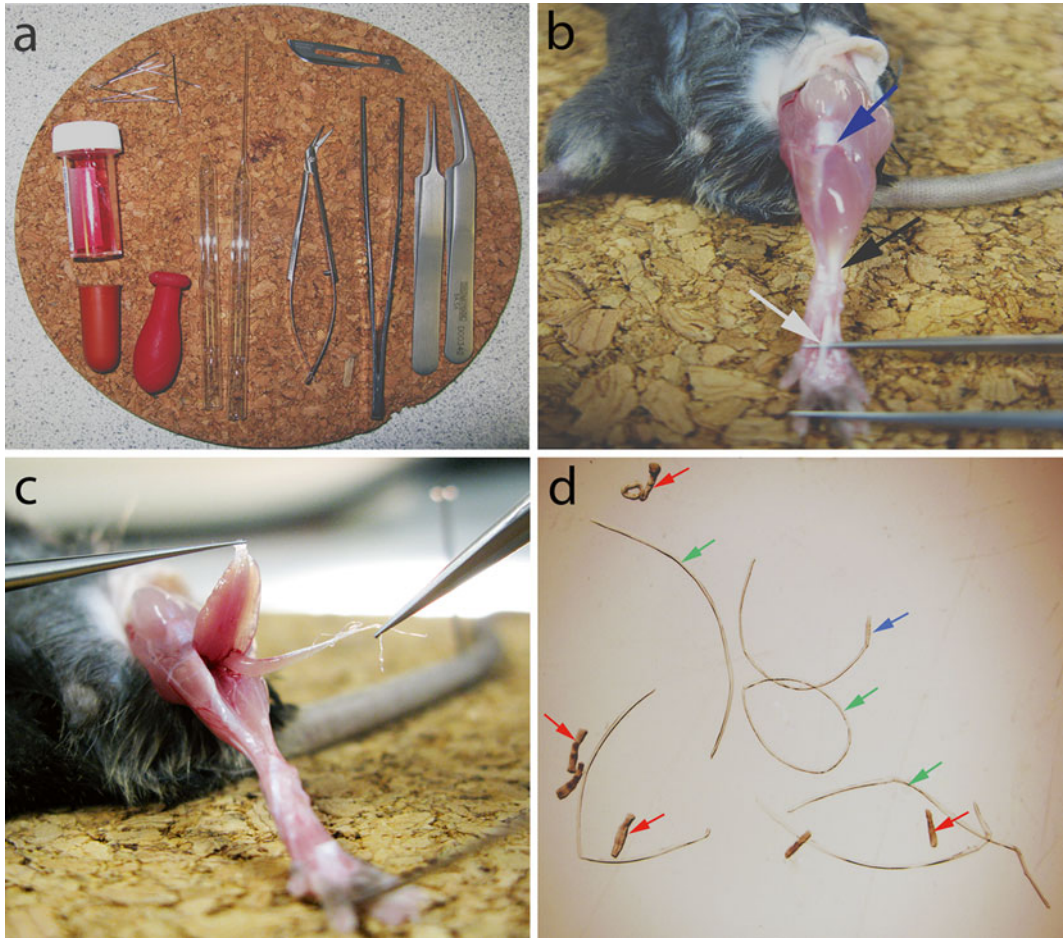


Fig. 1 Dissection and isolation of murine EDL myofibres (**a**) Instruments and equipment required for isolating myofibres. Clockwise from *top left*: corkboard, metal dissection pins, scalpel blade, 2× fine forceps, fine forceps with teeth, microscissors, glass Pasteur pipettes cut and heat-polished to give wide and small apertures, thin- and thick-walled rubber pipette bulbs and 7 ml bijou containing 0.2% collagenase/DMEM. (**b**) Mouse pinned in cross arrangement with skin removed for dissection of the left EDL. Distal EDL tendons have been lifted with forceps (indicated with a *white arrow*). The tendon lies on the tail side of the distal *tibialis anterior* tendon (indicated with a *black arrow*), and this is where the cut EDL tendons should be grasped and looped out. The approximate location of the proximal EDL tendons is indicated with a *blue arrow*. (**c**) Partially removed EDL muscle held by the four distal EDL tendons (*right*), with the *tibialis anterior* held to reveal relative locations (*left*). (**d**) Isolated myofibres before wash steps, with viable myofibres being hairlike and refractive (*green arrows*), and easily distinguishable from fully hypercontracted myofibres (*red arrows*) and myofibres with partial contraction (*blue arrow*)

dish. Place dishes in the 37 °C/5 % CO₂ incubator for at least 30 min to allow the DMEM to warm.

4. Score around a glass Pasteur pipette using a diamond pen and then snap away the end to create pipettes with diameters at the mouth of approximately 1, 4, and 6 mm (Fig. 1a).

5. Heat polish the cut ends of the Pasteur pipettes using a Bunsen burner to melt the glass at the mouth to remove the sharp and jagged edges. Test whether the edges are smooth by circling on a piece of aluminum foil; if the foil tears, it is not smooth enough and will damage fibres. Reheat carefully to sterilize and store in the tissue culture hood.
6. Immediately before dissection, weigh out collagenase and prepare as a 0.2 % solution in DMEM. Approximately 2 ml collagenase solution per EDL muscle is sufficient (*see Note 1*).
7. In the tissue culture hood, filter-sterilize the collagenase/DMEM using a sterile syringe with a 0.2 μ M filter and for each muscle, aliquot 2 ml of collagenase solution into a 7 ml bijoux tube. Place into a 37 °C water bath or incubator.

3.2 Dissection of the EDL Muscle from the Mouse Hindlimb

1. Euthanize the mouse by cervical dislocation (this method is preferable to CO₂ inhalation as it prevents muscles becoming hypoxic) (*see Note 2*).
2. Saturate the hind limbs and lower half of body with 70 % ethanol solution and closely shave the hind limbs using a scalpel. Rinse with 70 % ethanol and wipe with a tissue to remove shaved hair.
3. Pin the mouse onto a cork dissecting board, with one forelimb pinned through the palm. Pin the contralateral hindlimb through the dorsal side of the paw. Extend the tail under the pinned hindlimb to one side, and the contralateral hindlimb to the opposite side, to form a cross arrangement (Fig. 1b).
4. Carefully make an incision with a scalpel through the skin from above the knee to the paw stopping just proximal to the digits. Free the skin from the underlying muscle on both sides of the incision, by gripping the skin with toothed forceps and using another pair of forceps to ease the skin away from the underlying musculature to expose the *tibialis anterior*.
5. Alternatively, you can remove the skin from the leg musculature prior to pinning. Make an incision with the scalpel through the skin at the back of the leg, from above the knee to the heel. Grasp the skin on one side of the incision with the toothed forceps and pull towards the body of the mouse, removing the skin from the leg. This ensures that the musculature and tendons are not damaged prior to dissection.
6. Locate the four tendons of the EDL on the dorsal side of the paw, and cut them proximal to their insertions on the base of the third phalanx of digits two to five (Fig. 1b).
7. Gently cut/tear through the connective tissue overlying the *tibialis anterior* muscle, and par away using forceps.

8. Locate the EDL tendons proximal to the extensor retinaculum (annular ligament). They are lateral to the tendon of the *tibialis anterior*, on the same side as the extended tail (Fig. 1b). Gently loop out the tendons, thus pulling the cut ends through the extensor retinaculum. Ensure that all four tendons are present, and place clear of the *tibialis anterior* tendon.
9. Grip the distal *tibialis anterior* tendon using forceps just proximal to its insertion on the first cuneiform and proximal end of the first metatarsal. Cut the tendon with scissors distal to the point of holding. Firmly grip the *tibialis anterior* distal tendon and gently ease it away from the underlying musculature and bone, using forceps to gently disrupt connections, but avoiding the underlying EDL muscle.
10. Hold the *tibialis anterior* perpendicular to the leg and cut the muscle as close to the ventral crest of the tibia and knee joint as possible without touching the underlying musculature and remove (Fig. 1c).
11. Firmly grip all four distal tendons of the EDL muscle and ease the muscle away from the underlying musculature and bone, being careful not to pull the muscle excessively.
12. Move the musculature around the knee and locate two tendons to the side of the knee (Fig. 1b). Carefully cut the two proximal tendons where the muscle arises from the lateral epicondyle of the femur with fine scissors. The EDL muscle should then easily slide free with gentle pulling on the distal tendons. If not, ensure that the proximal tendons are completely sectioned (see Note 3). Handle the liberated EDL only by the distal tendons to avoid damage. Immediately transfer to a pre-warmed bijoux of collagenase/DMEM and label tube with a waterproof pen (see Note 4).
13. Re-pin the mouse using the contralateral forelimb and hindlimb into the cross configuration. Remove the second EDL by following steps 3–12 of Subheading 3.2 as above.

3.3 Isolation of Myofibres by Enzymatic Digestion and Trituration

1. Incubate the muscles in collagenase/DMEM in a shaking water bath or incubator for 90–120 min at 37 °C (occasionally mix the contents if using an incubator). As a general guide, the EDL muscle from a 6- to 12-week-old mouse requires approximately 90 min of digestion. However, the precise time depends upon both the age and size of the mouse and the activity of the batch of enzyme used, and should be determined empirically. Digestion is finished when the muscle looks less defined and slightly swollen, and under the microscope, hairlike single fibres are seen coming away from the edge of the muscle.

2. When digestion is complete, remove the bijoux containing the muscle from the water bath/incubator. If using a water bath, wipe dry, and then wipe with 70 % ethanol before placing in the culture hood. Also place one DMEM-containing deep Petri dish per muscle in the culture hood.
3. Fit the largest diameter heat-polished glass Pasteur pipette with a rubber pipette bulb and coat the inside with 5 % BSA solution just before use to prevent myofibres from adhering. Carefully remove the muscle from the bijoux and place into a deep Petri dish containing DMEM.
4. Place the deep Petri dish containing the digested muscles back into the incubator and allow the muscle to “rest” for approximately 20–30 min.
5. Place the stereo dissecting microscope directly in the tissue culture hood or lamina flow cabinet, together with the deep Petri dish containing the muscle and a second containing pre-warmed DMEM. If unable to place the microscope in the hood, position on a clean, draft-free bench.
6. The muscle should look swollen and less defined, with myofibres emanating from its edges. Using the largest diameter pre-rinsed heat-polished Pasteur pipette, take up the muscle and triturate repeatedly. Ensure that the muscle does not contact air. This procedure will result in highly refractive, hairlike fibres being liberated from the muscle (Fig. 1d). In addition, hypercontracted myofibres (Fig. 1d) together with fat droplets, tendon, and other debris will also be released.
7. Continue to dissociate myofibres from the muscle until it becomes clearly fragmented. Take up a small-diameter Pasteur pipette (fitted with another rubber pipette bulb) and pre-rinse with 5 % BSA solution (*see Note 5*). Carefully collect intact liberated fibres (Fig. 1d) and place them in a fresh dish of DMEM (the “collecting dish”), being careful to avoid collecting hypercontracted myofibres and debris.
8. At this point it may be helpful to remove the remaining muscle to another deep Petri dish and continue to triturate to isolate myofibres using a fire-polished, BSA rinsed, Pasteur pipette with a slightly smaller aperture. Continue to collect liberated myofibres using the fine-aperture Pasteur pipette. Eventually, there will be a clump of muscle from which no further viable myofibres are easily liberated. Do not allow the isolated muscle fibres to cool too much; return the dish to the incubator every 20 min for a minimum of 10 min. If both EDL muscles are being isolated, switch between muscles every 20 min (*see Note 6*).
9. Check whether the transferred myofibres are smooth and free of associated endothelium. Select with a fine-aperture Pasteur

pipette and serially transfer isolated myofibres through 1–2 further deep Petri dishes of DMEM to ensure that any contaminating endothelium, cells, and collagenase are removed.

10. Store dishes of cleaned myofibres in the incubator at 37 °C with 5 % CO₂. Fix myofibres as soon as isolation is complete (Subheading 3.5) to study satellite cells close to mitotic quiescence.

3.4 Non-adherent Myofibre Culture

Isolated myofibres can be cultured for at least 72 h, enabling satellite cells to be studied throughout early activation, cell cycle progression, and entry into differentiation.

1. Under the microscope, transfer clean myofibres into a BSA-coated deep Petri dish containing plating medium composed of DMEM supplemented with 10 % horse serum and 0.5 % chick embryo extract.
2. Culture myofibres in the incubator at 37 °C/5 % CO₂. As a guide, after 24 h virtually all satellite cells have activated to express MyoD, by 48 h most will have gone through the first cell division, and by 72 h, some are entering differentiation as revealed by myogenin expression (Fig. 2d) [10]. After ~96 h however, the myofibres tend to knot up and are hard to analyze by immunostaining.
3. To harvest, place the dish under the microscope and carefully remove myofibres for fixation as per 3.5. Once myofibres have been cultured for 48 h, satellite cells will have emerged from the basal lamina and started to proliferate, producing clusters of cells attached to the myofibre. These are delicate and easily detached, so care must be taken during fixation and immunostaining. At these later time points, it is better to remove most of the medium and gently add the 4 % PFA fixative directly to the myofibres in the deep Petri dish (*see* Note 7).

3.5 Fixing Isolated Myofibres and Associated Satellite Cells

1. Under the microscope, carefully collect myofibres using a heat-polished, BSA rinsed Pasteur pipette with a small aperture and place in a 2 ml clear round-bottomed microcentrifuge tube that has also been pre-rinsed with 5 % BSA/PBS. At least 25–30 myofibres are usually required per immunostaining. Label the microcentrifuge tube.
2. Stand the microcentrifuge tube upright and allow the myofibres to sink to the bottom.
3. Carefully remove the medium using a Pasteur pipette with a small aperture by gently running it just below the liquid surface until about 5 mm of medium remains above the myofibres.
4. Incline the microcentrifuge tube to a 45° angle and trickle pre-warmed 4 % PFA/PBS down the inside of the tube.

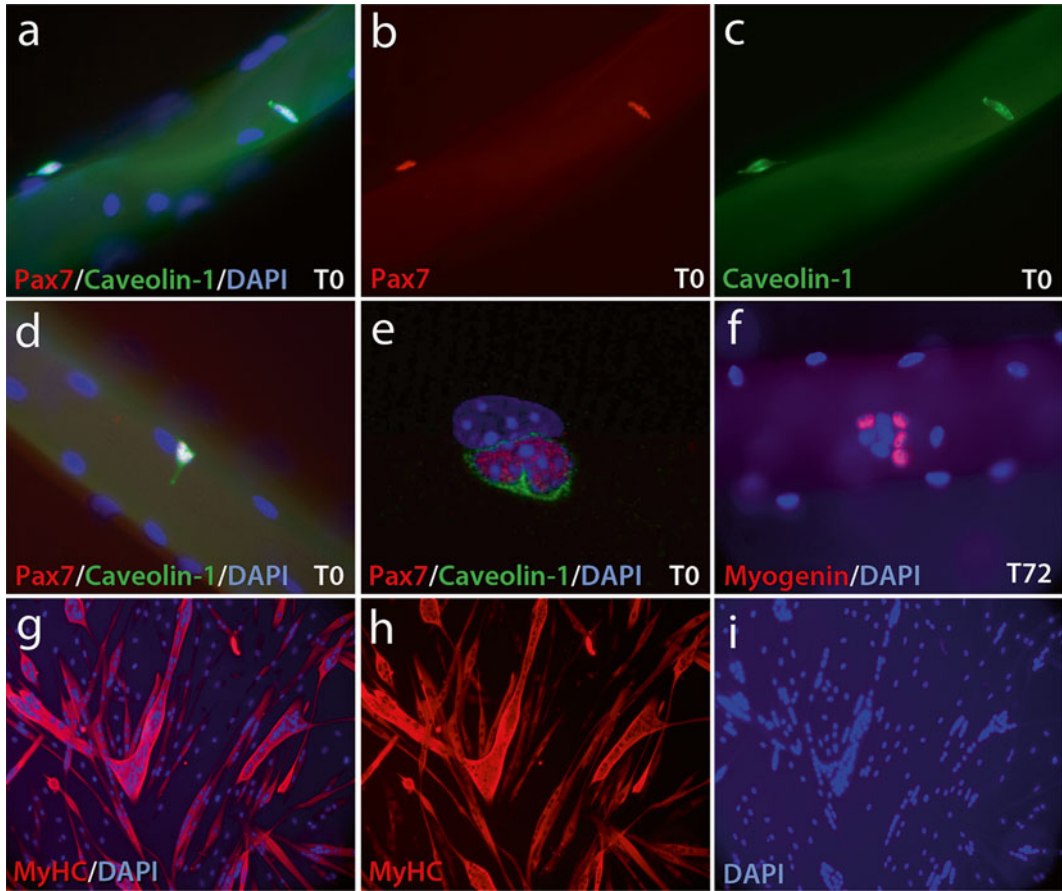


Fig. 2 Immunostained myofibres with associated satellite cells and satellite cell-derived myoblast cultures. (a–d) Freshly isolated myofibres with their associated quiescent satellite cells immunostained for Pax7 (red) and Caveolin-1 (green), with both satellite cell nuclei and myonuclei identified by DAPI (blue). (e) High-power confocal image of a satellite cell immunostained with Pax7 (red) and Caveolin-1 (green), counterstained with DAPI to highlight an adjacent myonucleus. (f) Myofibre cultured for 72 h by which time some satellite cell-derived myoblasts are entering myogenic differentiation, as revealed by immunostaining for myogenin (red), with DAPI counterstain. (g–i) Satellite cell-derived myoblasts from plated myofibre cultures seeded at high density and incubated in differentiation medium for 48 h, before immunostaining for myosin heavy chain (MyHC) (red) and counterstained with DAPI (blue)

Incubate the myofibres in 4 % PFA/PBS for 12 min with gentle agitation (e.g., on a tilt table).

5. Stand microcentrifuge tube to allow myofibres to settle and carefully remove the 4 % PFA/PBS, again leaving about 5 mm of liquid above the myofibres.
6. Gently wash the myofibres at least three times in 1.5 ml of PBS to remove PFA (see Note 7).
7. Store in the final wash of PBS in the microcentrifuge tube at 4 °C. Ideally, immunostain within 1–2 weeks.

3.6 Immunostaining Isolated Myofibres and Associated Satellite Cells

1. Place a microcentrifuge tube of fixed and washed myofibres upright, and allow the myofibres to settle to the bottom.
2. Remove the PBS with a pipette, replace with 0.5 % Triton-X 100 detergent/PBS, and incubate for 8–10 min at room temperature with gentle agitation to permeabilize the cell membranes of the myofibre and satellite cells.
3. Remove the 0.5 % Triton-X 100 detergent/PBS and add 10 % serum/PBS to block nonspecific antibody binding, and incubate for at least 30 min at room temperature with gentle agitation (e.g., on a tilt table). Choice of serum is dictated by the species that the secondary antibody was raised in.
4. Dilute primary antibodies to desired concentration in 0.035 % carrageenan/PBS. Remove block solution when myofibres have settled and add the primary antibody before incubating overnight at 4 °C with gentle agitation on a tilt table.
5. Stand microcentrifuge tube upright and remove the primary antibody once myofibres have settled (this can be stored at 4 °C and reused) and wash the myofibres three times for 5 min in PBS containing 0.025 % Tween-20.
6. Dilute fluorochrome-conjugated secondary antibodies in 0.035 % carrageenan/PBS and incubate myofibres for 60 min at room temperature with gentle agitation, protected from light.
7. Stand the microcentrifuge tube upright, once the myofibres have settled, remove the secondary antibody (this can be stored at 4 °C and reused) and wash the myofibres three times for 5 min in 0.025 % Tween-20/PBS.
8. Outline several glass slides with a liquid repellent pap pen. This will prevent any myofibres from slipping off during mounting. Slides chemically treated to increase adherence can also be used.
9. Pre-rinse a clean heat-polished round-ended Pasteur pipette (fitted with a rubber pipette bulb) with 5 % BSA solution. Empty myofibres into a pre-rinsed dish. Under the microscope carefully collect myofibres and transfer them onto the glass slide. After several muscle fibres have been transferred, remove as much liquid from the glass slide as possible to help myofibres adhere. Continue until all myofibres are in place, and again remove excess liquid.
10. Place a couple of drops of mounting medium (such as Vectashield containing DAPI) on the slide, and gently lower a 50 mm×22 mm cover slip, being careful not to trap air bubbles or wash myofibres off the slide. A small amount of nail varnish can be brushed along the edge of the cover slip to secure it (*see Note 8*). Wait 10 min for the nail varnish to dry, protected from light.

Examples of freshly isolated satellite cells immunostained for Pax7 and Caveolin 1 are illustrated in Fig. 2a–c. A myofibre that has been in culture for 72 h to allow the satellite cells to activate, proliferate, and enter differentiation, and then immunostained for Myogenin, is illustrated in Fig. 2f (*see Note 9*).

3.7 Plated Satellite Cell Culture and Immunostaining

Myofibres can also be plated to allow their associated satellite cells to migrate onto the tissue culture substrate. Large quantities of satellite cell-derived myoblasts can be obtained using this technique. It also allows the study of satellite cell-derived myoblast differentiation and fusion into large multinucleated myotubes.

1. Defrost Matrigel stock overnight at 4 °C. On ice, dilute Matrigel to 1 mg/ml in DMEM and aliquot into 2 ml microcentrifuge tubes. It is essential to complete this step on ice or the Matrigel will form lumps. Aliquots of diluted Matrigel can be stored at 4 °C for up to 2 weeks, or frozen at -20 °C for longer term storage.
2. Rinse a 6-well tissue culture dish with Matrigel, ensuring that the surface has been completely coated. Immediately replace the Matrigel to 4 °C. Place the 6-well plate in an incubator at 37 °C/5 % CO₂ for 45 min to allow the Matrigel to gel.
3. Prepare proliferation medium by supplementing DMEM with 20 % foetal bovine serum, 10 % horse serum, 1 % chick embryo extract, and 1/10,000 bFGF. Pre-warm the medium in a water bath at 37 °C before plating 2 ml per well of the Matrigel-coated 6-well plate.
4. Coat a heat-polished fine-aperture glass Pasteur pipette with 5 % BSA/PBS and transfer approximately 100 freshly isolated myofibres into each well of the Matrigel-coated 6-well plate. Ensure that the myofibres are evenly spaced across the well. Return to the incubator and culture undisturbed for 72 h, since the myofibres are easily dislodged.
5. After 72 h, satellite cells will have activated and migrated from the myofibres. Under a stereo dissecting microscope, gently remove attached myofibres using a 1 ml pipette by agitating the medium over a myofibre. Once myofibres are detached, remove medium and wash cells with 1 ml of PBS per well.
6. Trypsinize satellite cells using 500 µl trypsin-EDTA for 3–5 min in a humidified incubator at 37 °C/5 % CO₂. Once the cells have detached, resuspend in 5 ml of plating medium.
7. Transfer the satellite cell suspension to a sterile non-coated 60 mm Petri dish and incubate at 37 °C/5 % CO₂ for 15 min. This pre-plating step will remove any contaminating fibroblasts, as they readily adhere to the plastic culture dish.

8. Remove the medium containing the non-adhered satellite cells from the Petri dish and plate in proliferation medium at the desired density on fresh Matrigel-coated plates.
9. Satellite cells can be passaged 3–4 times and will remain in a proliferative state for up to 1 week, but will then begin to differentiate.
10. Coat each well of a Labtech 8-well chamber slide with Matrigel and allow to gel at 37 °C for 30 mins. Add satellite cells and either 200 µl of proliferation or differentiation medium (DMEM with 2 % horse serum). In differentiation medium, satellite cells will start to differentiate immediately, with large myotubes forming by 48 h. If satellite cells are being differentiated for longer periods, carefully replace the medium every 48 h (*see* **Note 10**).
11. To fix plated satellite cells in chamber slides, remove most of the medium and add 200 µl of pre-warmed 4 % PFA/PBS for 15 min. Wash chamber slides three times with 500 µl of PBS to remove PFA. Differentiated muscle cultures are especially prone to detaching from the dish unless the fixation and staining steps are carefully undertaken (*see* **Note 7**).
12. The process for immunostaining plated satellite cell cultures is the same as with floating myofibre immunostaining (**steps 2–7** Subheading 3.6).
13. After the final 0.025 % Tween 20/PBS wash, invert and outline the wells with a marker pen, and then remove the walls of the chamber slide and plastic gasket. Pipette a small amount of Vectashield mounting medium containing DAPI onto the slide and carefully place a glass 50 × 22 mm cover slip on top. Brush the edges of the cover slip with a small amount of nail varnish and leave to dry protected from light for 10 min.
14. Plated satellite cell-derived myoblasts that have differentiated into large multinucleated myotubes are illustrated in Fig. 2g–i.

4 Notes

1. It is important to batch test replacement reagents, such as collagenase, against existing, optimized components. There are variable amounts of proteases in batches of collagenase. Try to obtain collagenase with approximately 53 U of neutral protease and 0.6 U of clostripain [28].
2. Dissect the EDL muscle as soon as possible after euthanasia. This will ensure that the muscle is healthy and improve the ease of the dissection.
3. Ensure the complete removal of the connective tissue from the overlying musculature using the forceps before removing the

tibialis anterior, and continue to par away if any becomes caught during the removal process. Failure to do this may result in the requirement of extra force to free the EDL muscle, which may damage it. Never touch the EDL muscle directly, and only manipulate it via the tendons. Study the EDL before placing in collagenase; if the muscle does not have both proximal and distal tendons, the muscle may be damaged, and few viable myofibres may then be isolated..

4. EDL muscles can be stored in DMEM (without collagenase) for transportation etc, but have never left them for more that ~90 mins.
5. Different thicknesses of rubber pipette bulbs allow differing degrees of sensitivity; thin-walled bulbs are useful for the dissociation of the muscle requiring bigger volumes to be moved, whereas thick-walled bulbs are better for transferring individual myofibres in smaller volumes.
6. Do not allow muscles and myofibres to cool when out of the incubator—switch between muscles every 15–20 min.
7. During fixation and staining of satellite cells, dribble solutions carefully down the inside wall of the deep Petri dish/microcentrifuge tube, rather than directly onto the myofibres/cells to prevent cells from detaching.
8. Brush a small amount of nail varnish around the edges of the cover slip. This will dry faster than the mounting medium, and prevent cover slips from moving.
9. There are several markers for identifying quiescent satellite cells by immunohistochemistry; most widely used is mouse monoclonal anti-Pax7, but CD34, Caveolin-1, and the calcitonin receptor also have good commercially available antibodies. Mouse monoclonal anti-MyoD (clone 5.8) can be used to detect activated satellite cells, and mouse monoclonal anti-myogenin (F5D) for identifying cells entering differentiation. [30, 34, 35].
10. Approximately 5,000 satellite cells per well of a Labtech 8-well chamber slide is appropriate for studying proliferation, or 25,000 per well for differentiation.

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Human Neural Crest Stem Cells Derived from Human Pluripotent Stem Cells

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Abstract

The neural crest cells give rise to neurons and glia in the peripheral nervous system, which is an important component of the nervous system. Here we developed a scalable process of inducing neural crest stem cells (NCSCs) from hESCs/iPSCs by a combination of growth factors in medium conditioned on stromal cells, and showed that NCSCs could be purified by p75 using FACS. In vitro-expanded NCSCs were able to differentiate into neurons and glia (Schwann cells) of the peripheral nervous system (PNS) as well as mesenchymal derivatives.

Key words Neural crest, Human embryonic stem cells, Differentiation, Schwann cells, Peripheral neuron

1 Introduction

The neural crest (NC) is a transient, multipotent, migratory cell population unique to vertebrates that gives rise to a diverse cell lineage. Much of our knowledge on NC development comes from studies of organisms such as chicken and zebrafish as human NC is difficult to obtain because of its transient nature and limited availability of human fetal cells [1].

Several key steps and signaling pathways involved in NC development (e.g., Wnt and TGF β) have been identified and shown to be conserved across various species [2–5]. Evidence that these pathways are likely conserved in humans has come from analyzing hereditary disorders including neurocristopathies and peripheral neuropathies that affect different lineages in the PNS. It appears that the Sox family of genes is important for NC induction and self-renewal [6], while c-kit/SCF and endothelin are important for melanocyte differentiation, neurogenins for sensory neuron differentiation [7], MASH and HAND genes for autonomic differentiation [8], neuregulins for Schwann cells, and RET/GDNF for enteric neurons [9].

Several research groups have reported NC differentiation from hESCs; most of the previously described methods used a continuous differentiation protocol to generate NC lineages (e.g., sensory neurons and Schwann cells) directly from pluripotent cells [10–13]. Such methods are difficult to scale up and in general take many weeks if not months to obtain a certain cell type and with limited purity. We have focused our effort on developing a method/medium that can arrest the cells at the NCSC stage in an attempt to improve differentiation efficiency and enable to scale up.

To address this issue, we developed a stage-specific differentiation process that can be broken into the following steps: (1) induction of migrating NCSCs that expresses NC markers p75, Sox 9, and Sox10; (2) purification of NCSCs by FACS with p75; (3) in vitro culture/expansion of NCSCs; and (4) differentiation of NCSCs to various cell types. Each of these steps can utilize a medium with a combination of growth factors as well as a defined substrate. We showed that NC cells could be efficiently induced from hESCs by a combination of growth factors in medium conditioned on stromal cells, and that NC stem cells (NCSCs) could be purified by p75 using FACS. FACS-isolated NCSCs could be propagated in vitro for five passages and cryopreserved while maintaining the NCSC identity characterized by the expression of a panel of NC markers such as p75, Sox9, Sox10, CD44, and HNK1. In vitro-expanded NCSCs were able to differentiate into neurons and glia (Schwann cells) of the peripheral nervous system (PNS) as well as mesenchymal derivatives. Using a defined medium we were able to generate and propagate a nearly pure population of Schwann cells that uniformly expressed GFAP, S100, and p75 [14].

2 Materials

2.1 Equipment

1. Biological safety cabinet.
2. Cell culture incubator.
3. Light microscope.
4. Fluorescence microscope.
5. Centrifuge.

2.2 Supplies

1. 35, 60 mm polystyrene dish.
2. 4-Well plates.
3. 15, 50 mL Polystyrene conical tube.
4. 5, 10, and 25 mL Serological pipettes.
5. 60×15 mm Petri dish.
6. 100, 500 mL filter unit.
7. Cryogenic vials.

2.3 Media and Reagents

1. Medium for maintenance of hESC and iPSC (100 mL): 75 mL knockout Dulbecco's modified Eagle's medium/Ham's F12, 20 mL knockout serum replacement, 1 mL MEM nonessential amino acids (2 mM), 2 mL L-glutamine (4 mM), 100 μ L β -mercaptoethanol (0.1 mM). Sterile filter with a 0.22 μ m filter, and add bFGF (final concentration is 4 ng/mL) just prior to feeding cells. Medium is stored at 4 °C for up to 1 week.
2. Medium for culture of hESC and iPSC under defined conditions (100 mL): 90.8 mL DMEM/F-12 + GLUTAMAX™ (1 $\times$), 7.2 mL BSA (25 %): Sterile filter with a 0.22 μ m filter, and add 80 μ L bFGF (final concentration is 8 ng/mL), 2 mL STEMPRO hESC SFM growth supplement (50 $\times$), and 182 μ L 2-mercaptoethanol just prior to feeding cells. Medium is stored at 4 °C for up to 1 week.
3. Neural stem cell medium (NSC medium, 100 mL): Combine Neurobasal (96 mL), 1 mL MEM nonessential amino acids (2 mM), 1 mL GlutaMAX-I CTS, and 2 mL B27 (50 $\times$). Sterile filter with a 0.22 μ m filter, and add bFGF (final concentration is 20 ng/mL) just prior to feeding cells. Medium is stored at 4 °C for up to 1 week.
4. PA6 conditioned medium: Medium conditioned by the stromal cell line PA6 as described before [15].
5. Neural crest stem cell induction medium (NC medium, 100 mL): 50 mL NSC medium and 50 mL medium conditioned by the stromal cell line PA6 with Rock inhibitor (10 μ M) and ascorbic acid (200 μ M).
6. NCSC-FREEZE-A medium (100 mL): 100 mL NC medium without Rock inhibitor and ascorbic acid.
7. NCSC-FREEZE-B medium (100 mL): 20 mL DMSO, 80 mL NC medium without Rock inhibitor and ascorbic acid.
8. Medium for Schwann cell differentiation (100 mL): 100 mL complete Mensen PRO RS™ medium supplemented with 100 μ L Heregulin- β 1 (20 ng/mL).
9. Medium for peripheral neuronal differentiation (100 mL): 98 mL Neurobasal medium supplemented with 2 mL B27 (1 $\times$), BDNF (10 ng/mL), ascorbic acid (200 μ M), NGF (10 ng/mL), and dcAMP (0.1 mM). After 5 days of differentiation, the dcAMP was withdrawn.
10. Medium for non-neural differentiation.
11. Complete osteogenesis differentiation medium: Prepare according to the manufacturer's instructions.
12. Complete chondrogenic differentiation medium: Prepare according to the manufacturer's instructions.
13. Complete adipogenic differentiation medium: Prepare according to the manufacturer's instructions.

14. 10 µg/mL recombinant human FGF2 dissolved in sterilized PBS with 0.1 % BSA, stored at -20 °C.
15. 20 µg/mL recombinant human Heregulin-β1, dissolved in sterilized PBS with 0.1 % BSA, stored at -20 °C.
16. 50 µg/mL recombinant human BDNF and GDNF, dissolved in sterilized PBS with 0.1 % BSA, stored at -20 °C.
17. 200 mM Ascorbic acid, dissolved in 352 mg ascorbic acid in 10 mL PBS, stored at -20 °C.
18. 10 µg/mL recombinant human nerve growth factor (NGF), dissolved in sterilized PBS with 0.1 % BSA, stored at -20 °C.
19. 10 mM Stemomolecule Y27632 (Rock inhibitor) reconstituted in DMSO, stored at -20 °C.

2.4 Antibodies

1. Rabbit anti-SOX10, Abcam, ab25978-100.
2. Rabbit anti-SOX9, Santa Cruz, SC20095.
3. Mouse anti-Nestin, BD Transduction laboratories, 611658.
4. Rabbit anti-P75, Abcam, ab52987.
5. Mouse anti-CD271-PE, MiltenyiBiotec, 130-091-885.
6. Mouse anti-Peripherin, Chemicon, MAB1527.
7. Rabbit anti-Brn3a, Chemicon, Ab5945.
8. Mouse anti-S100b, Sigma, S2532.
9. Rabbit anti-βIII-tubulin isotype III clone SDL.3D10, Sigma, T8660.
10. Rabbit anti-GFAP, Chemicon, M0761.
11. Rabbit anti-CD44, Abcam, ab24504.
12. Rabbit anti-DBH, Chemicon, Ab1585.
13. Mouse anti-HNK1, Sigma, C0678.
14. Rabbit anti-tyrosine hydroxylase, Pel-Freeze, P40101.
15. Alexa Fluor 488 goat anti-mouse, Molecular Probes, A-11001.
16. Alexa Fluor 488 goat anti-rabbit, Molecular Probes, A-11008.
17. Alexa Fluor 594 goat anti-rabbit, Molecular Probes, A-11005.
18. Alexa Fluor 594 goat anti-mouse, Molecular Probes, A-11012.
19. Alexa Fluor 594 goat anti-goat, Molecular Probes, A-110080.
20. Alexa Fluor 486 goat anti-rat, Molecular Probes, A-11006.
21. Alexa Fluor 594 goat anti-rat, Molecular Probes, A-11007.

2.5 Media

1. Medium for maintenance of hESC and iPSC (100 mL): 75 mL knockout Dulbecco's modified Eagle's medium/Ham's F12, 20 mL knockout serum replacement, 1 mL MEM nonessential

amino acids (2 mM), 2 mL L-glutamine (4 mM), 100 μ L β -mercaptoethanol (0.1 mM). Sterile filter with a 0.22 μ m filter, and add bFGF (final concentration is 4 ng/mL) just prior to feeding cells. Medium is stored at 4 °C for up to 1 week.

2. Medium for culture of hESC and iPSC under defined conditions (100 mL): 90.8 mL DMEM/F-12 + GLUTAMAX™ (1 $\times$), 7.2 mL BSA (25 %). Sterile filter with a 0.22 μ m filter, and add 80 μ L bFGF (final concentration is 8 ng/mL), 2 mL STEMPRO hESC SFM growth supplement (50 $\times$), and 182 μ L 2-mercaptoethanol just prior to feeding cells. Medium is stored at 4 °C for up to 1 week.
3. Neural stem cell medium (NSC medium, 100 mL): Combine Neurobasal (96 mL), 1 mL MEM nonessential amino acids (2 mM), 1 mL GlutaMAX-I CTS, and 2 mL B27 (50 $\times$). Sterile filter with a 0.22 μ m filter, and add bFGF (final concentration is 20 ng/mL) just prior to feeding cells. Medium is stored at 4 °C for up to 1 week.
4. PA6 conditioned medium: Medium conditioned by the stromal cell line PA6 as described before [15].
5. Neural crest stem cell induction medium (100 mL NC medium): 50 mL NSC medium and 50 mL medium conditioned by the stromal cell line PA6 with Rock inhibitor (10 μ M) and ascorbic acid (200 μ M).
6. NCSC-FREEZE-A medium (100 mL): 100 mL NC medium without Rock inhibitor and ascorbic acid.
7. NCSC-FREEZE-B medium (100 mL): 20 mL DMSO, 80 mL NC medium without Rock inhibitor and ascorbic acid.
8. Medium for Schwann cell differentiation (100 mL): 100 mL complete MensenPRO RS™ medium supplemented with 100 μ L Heregulin- β 1 (20 ng/mL).
9. Medium for peripheral neuronal differentiation (100 mL): 98 mL Neurobasal medium supplemented with 2 mL B27 (1 $\times$), BDNF (10 ng/mL), ascorbic acid (200 μ M), NGF (10 ng/mL), and dcAMP (0.1 mM). After 5 days of differentiation, the dcAMP was withdrawn.
10. Medium for non-neural differentiation.
11. Complete osteogenesis differentiation medium: Prepare according to the manufacturer's instructions.
12. Complete chondrogenic differentiation medium: Prepare according to the manufacturer's instructions.
13. Complete adipogenic differentiation medium: Prepare according to the manufacturer's instructions (*see Note 1*).

2.6 Buffers for Immunocyto- chemistry

1. Cell fixation buffer (100 mL): 1× PBS and 4 g paraformaldehyde.
2. Blocking buffer (100 mL): 95 mL 1× PBS, 10 mL goat serum, 10 mL 10 % BSA, and 0.1 mL Triton-X.
3. Antibody buffer (100 mL): 97 mL 1× PBS, 8 mL goat serum, 10 mL 10 % BSA, and 0.1 mL Triton-X.

3 Methods

3.1 Coating of Cell Culture Dishes

3.1.1 Geltrex-Coated Dishes

Incubate cell culture dishes in Geltrex substrate solution (1:50) in DMEM/F12 at room temperature for at least 1 h. Use 1.5 mL per 35 mm dish and 3 mL per 60 mm dish. Coat dishes fresh prior to cell culture (optionally, store coated dishes at 4 °C for up to 7 days).

3.1.2 PO/ Lam-Coated Dishes

1. Incubate cell culture dishes with solution of poly-ornithine in cell culture-grade water (f.c. 20 µg/mL), ON at 4 °C (optionally 2 h, 37 °C).
2. Rinse twice with cell culture-grade water.
3. Add 2 mL of laminin solution in cell culture-grade water (f.c. 10 µg/mL) and incubated ON at 4 °C (optionally 2 h, 37 °C).

3.2 hESC/iPSC Cultures Under Defined Conditions

1. At least 1–2 h prior to cell culture, coat culture dishes with solution of Geltrex substrate.
2. If differentiated colonies are present on a confluent paternal dish, remove them by scraping off excess differentiated fragments with a pipette tip, syringe needle, or a tool made of a heat-stretched glass Pasteur pipette (under sterile conditions).
3. Aspirate hESC complete medium from the dish together with the previously differentiated cells scraped, and wash once with pre-warmed DMEM/F12.
4. Add 3 mL of 1 mg/mL Collagenase buffer. Incubate for 50–55 min at 37 °C in cell culture incubator. Collect colonies in 15 mL tube. Wash with DMEM/F12 until colonies detach. Centrifuge colonies for 2 min at 180×g. Make sure that the colonies are in a pellet not floating.
5. Add 4–5 mL hESC complete medium and pipette all of the ESC colonies gently.
6. Transfer equal aliquots from parental dish to 4–5 new dishes coated with Geltrex and containing fresh hESC complete medium. Distribute ESC colonies evenly and place dishes in a cell culture incubator.
7. Change media daily (*see Note 3*).

3.3 Induction of Neural Crest Stem Cells from hESCs/ iPSCs

1. When hESCs/iPSCs are confluent, aspirate ESC medium off ESCs and rinse cells with pre-warmed DMEM/F12. Wash 2–3 times with DMEM/F12.
2. Add 3 mL of 1 mg/mL Collagenase buffer. Incubate for 50–55 min at 37 °C. Collect colonies in 15 mL tube. Wash with DMEM/F12 until colonies detach. Centrifuge colonies for 2 min at 180×*g*. Make sure that colonies are in a pellet and not floating.
3. Add 3 mL hESC complete medium and pipette all of the ESC colonies gently. Transfer the entire content into Petri dish for EB formation.
4. For induction of neural crest stem cells (day 0), the next day change medium using NC medium and culture EBs in suspension for 10 days in NC medium on a rocking platform. Change medium every other day (5 mL/60 mm dish) (*see Note 4*).

3.4 Purification of Neural Crest Stem Cells from hESCs/ iPSCs

1. Induce attachment by plating EBs on a Geltrex-coated 60 mm cell culture dish. When plating EBs, make sure that there is enough space for colonies to grow out without contacting one another. Approximately 20–30 EBs should be suspended in NC medium and plated onto culture dish. Distribute EBs evenly and place the dish in cell culture incubator. EBs should attach overnight. Change medium every other day.
2. Four days after differentiation, dissociate cells using accutase and pass through a 70 µm cell strainer.
3. Centrifuge harvested cells for 3 min at 180×*g* at room temperature and aspirate supernatant carefully. Mechanically triturate cells in NC medium.
4. Label triturated cells with antibody (p75) for flow cytometry. After 20-min incubation with anti-CD271 (p75, NGFR/NTR)-PE (1 µl per million cells) on ice in the dark, wash cells once in staining medium. Centrifuge for 3 min at 180×*g* at room temperature and aspirate the supernatant. Resuspend cells in staining medium to obtain density of 3×10^6 cells/mL (*see Note 2*).
5. Perform cell sorting by flow cytometry. Samples were sorted on a BD FACSAria Special Order System by the FACSDiva 6.1.1 software. Collect p75+ cells for further culture.
6. Centrifuge p75+ cells and plate onto culture dishes coated with Geltrex in NC medium.
7. Expand NCSCs in NC medium as described below. Confirm NCSC identity by immunocytochemistry using antibodies against markers characteristic for neural crest stem cells. They will stain positive for the definitive NC markers p75 and HNK1 (*see Note 5*).

3.5 In Vitro

Expansion

and Cryopreservation of Neural Crest Stem Cells (NCSCs)

3.5.1 Enzymatic Passage of Neural Crest Stem Cells

1. Carefully aspirate medium from 60 mm dish containing confluent NCSCs.
2. Add 1–2 mL of pre-warmed accutase into the dish and place it in the cell culture incubator and for 3–5 min until cells detach.
3. Wash off NCSCs from the dish using P-1000 pipette and place cells in 15 mL conical tube. Rinse the dish once again with 3 mL of pre-warmed neurobasal medium and collect in the same tube.
4. Centrifuge cells at $180\times g$ for 3 min.
5. Aspirate supernatant carefully and resuspend cells in 3 mL of NC medium.
6. Aspirate Geltrex substrate solution from freshly coated dishes, add 4 mL of NC medium into each plate, and transfer 1 mL of NCSCs (from **step 5**) into each plate.
7. Distribute NCSCs evenly.
8. Place dishes in cell culture incubator and change medium every other day (*see Note 6*).

3.5.2 Cryopreservation of Neural Crest Stem Cells

1. Perform **steps 1–4** as described in Subheading [3.5.1](#).
2. Resuspend cells in a small volume of NCSC-FREEZE-A medium (1 mL or more if multiple dishes are combined) and count them.
3. Dilute cells using the same medium to obtain desired cell density (4–5 million cells/mL).
4. Carefully add an equal volume of NCSC-FREEZE-B medium containing 20 % DMSO under constant swirling (final cell density should be approximately 2×10^6 cells/mL). Mix gently 2–3 times by pipetting.
5. Aliquot into cryogenic vials (1 mL/vial).
6. Immediately freeze at $-80\text{ }^{\circ}\text{C}$ using isopropanol contraption and transfer vials into liquid nitrogen tank the following day.

3.5.3 Thawing Neural Crest Stem Cells from Frozen Stocks

1. Thaw cells in $37\text{ }^{\circ}\text{C}$ water bath.
2. Transfer cells to 15 mL conical tube containing 5 mL of pre-warmed NSC medium under constant swirling. Wash the cryovial with additional 1 mL of medium and transfer to the same 15 mL tube.
3. Centrifuge cells at $180\times g$ for 3 min.
4. Aspirate supernatant and resuspend cells in 5 mL of NC medium.
5. Plate onto Geltrex-coated dishes (2 million/35 mm dish).
6. Distribute evenly and place dishes in cell culture incubator.
7. Change medium every other day.

3.6 Immunocyto-chemistry

We routinely use single, double, or triple labeling to assess various marker expression profiles at different stages of neural crest stem cells and their derivatives. Below is an example of a double labeling of Nestin and SOX9.

1. Aspirate medium from dish and wash three times with PBS.
2. Add 4 % paraformaldehyde to cover neurons for 10 min.
3. Wash the cells three times with PBS.
4. Add 10 % goat serum in 0.1 % PBS/Triton-X for 20 min for blocking.
5. Prepare primary antibodies in 8 % goat serum in 0.1 % PBS/Triton-X (mouse anti-Nestin 1:1,000, rabbit anti-SOX9 1:250).
6. Aspirate blocking reagent, then add primary antibody solution, and incubate overnight at 4 °C.
7. Aspirate the primary antibody solution and wash three times with PBS.
8. Prepare secondary antibodies in 8 % goat serum in 0.1 % PBS/Triton-X (Alexa Fluor 488 goat anti-mouse and Alexa Fluor 594 goat anti-rabbit, both at dilution 1:1,000).
9. Add secondary antibody solution for 1 h in the dark at room temperature.
10. Wash three times with PBS.
11. Add 15 µL of ProLongantifade reagent with Hoechst on the cell layer and carefully place a cover slip on the top avoiding generation of bubbles. Keep in the dark at 4 °C until viewing.
12. Observe the staining under a fluorescence microscope with appropriate filter combinations.

3.7 Schwann Cell Differentiation

1. Evenly plate NCSCs onto a 35 mm poly-ornithine/laminin-coated dish at approximately 50 % density in 2 mL of NC medium and place the dish in cell culture incubator.
2. On the second day, aspirate the NCSC medium and replace it with 2 mL of Schwann cell differentiation medium. Differentiate NCSCs in Schwann cell differentiation medium for 40 days with medium change every other day.
3. Passage the cells when confluent (*see Note 7*).

3.8 Peripheral Neuron Differentiation

1. Evenly plate NCSCs onto a 35 mm poly-ornithine/laminin-coated dish at approximately 80 % density in 2 mL of NC medium and incubate ON at 37 °C, 5 % CO₂.
2. On the second day, aspirate off the NCSC medium and replace it with 2 mL of NB medium supplemented with B27 (1×), BDNF (10 ng/mL), ascorbic acid (200 µM), NGF (10 ng/mL), and dcAMP (0.1 mM).

3. After differentiation for 5 days, withdraw the dcAMP and differentiate the cells continuously for 3–4 weeks with the medium change every other day.
4. Passage the cells when confluent.

3.9 Non-neural Differentiation

3.9.1 Osteogenic Differentiation

1. Evenly plate NCSCs onto 35 mm Geltrex-coated dishes at approximately 80 % density in 2 mL of NC medium and incubate ON at 37 °C, 5 % CO₂.
2. On the second day, aspirate the NCSC medium and replace it with 2 mL of Mesen Pro medium.
3. After 2 weeks of differentiation, add complete osteogenesis differentiation medium and differentiate the cells continuously for 4 weeks with a medium change every other day.
4. Assess the differentiation by Alizarin Red S staining.

3.9.2 Chondrogenic Differentiation

1. To start chondrogenic differentiation, evenly plate NCSCs onto 35 mm Geltrex-coated dishes at approximately 80 % density in 2 mL of NC medium and incubate ON at 37 °C, 5 % CO₂.
2. On the second day, aspirate the NCSC medium and replace it with 2 mL of Mesen Pro medium. After 2 weeks of differentiation, culture a desired number of cells (250,000 per pellet) as pellets in 15 mL conical tubes in complete chondrogenic medium with a medium change every 3–4 days (*see Note 7*).
3. After 4 weeks of culture, fix cells with 4 % formaldehyde for 30 min and assess chondrogenic differentiation by immunohistochemistry (Alcian blue).

3.9.3 Adipogenic Differentiation

1. Evenly plate NCSCs onto 35 mm Geltrex-coated dishes at approximately 50 % density in 2 mL of NC medium and incubate ON at 37 °C, 5 % CO₂.
2. On the second day, aspirate the NCSC medium and replace it with 2 mL of Mesen Pro medium.
3. After 2 weeks of differentiation, culture cells in complete adipogenesis differentiation medium for 4 weeks with a medium change every 3–4 days.
4. After 4 weeks of culture, fix cells with 4 % formaldehyde for 30 min and assess adipogenic differentiation by oil red O staining.

4 Notes

1. All materials and reagents for cell culture must be sterile. All media and solutions must be pre-warmed to 37 °C. Media with growth factors should be used within 1 week.

2. All incubations are performed in a humidified 37 °C, 5 % CO₂, incubator.
3. Cells cultured under this condition for >20 passages maintain normal karyotype and remain pluripotent.
4. To form EBs, pipette ESC/iPSC colonies 3–5 times to break them into smaller clusters but do not overtriturate colonies. To change medium, transfer EBs to 15 mL conical tube for sedimentation (do not centrifuge). Aspirate medium and carefully resuspend EBs in fresh medium. Transfer EBs into the original Petri dish.
5. NCSCs can be expanded in the NC medium for at least five passages and used for differentiation or other analysis.
6. Cells from one confluent 60 mm dish shall be split onto three new 60 mm dishes (split 1:3). Approximately 2–2.5 million of cells per 60 mm dish will give a desirable density.
7. Schwann cell differentiation is not observed during early stages of NCSC culture. We can observe the GFAP⁺ cells only in the aged NCSCs cultured for more than 30 days.

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Dental Pulp Stem Cell (DPSC) Isolation, Characterization, and Differentiation

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Abstract

Dental pulp stem cells (DPSC) have been proposed as an alternative to pluripotent stem cells to study multilineage differentiation in vitro and for therapeutic application. Standard culture media for isolation and expansion of stem cells includes animal sera or animal-derived matrix components (e.g., Matrigel®). However, animal-derived reagents raise significant concerns with respect to the translational ability of these cells due to the possibility of infection and/or severe immune reaction. For these reasons clinical grade substitutes to animal components are needed in order for stem cells to reach their full therapeutic potential. In this chapter we detail a method for isolation and proliferation of DPSC in a chemically defined medium containing a low percentage of human serum. We demonstrate that in this defined culture medium a 1.25 % human serum component sufficiently replaces fetal bovine serum. This method allows for isolation of a morphologically and phenotypically uniform population of DPSCs from dental pulp tissue. DPSCs represent a rapidly proliferating cell population that readily differentiates into the osteoblastic, neuronal, myocytic, and hepatocytic lineages. This multilineage capacity of these DPSCs suggests that they may have a more broad therapeutic application than lineage-restricted adult stem cell populations such as mesenchymal stem cells. Further the culture protocol presented here makes these cells more amenable to human application than current expansion techniques for other pluripotent stem cells (embryonic stem cell lines or induced pluripotent stem cells).

Key words Dental pulp stem cells, Adult stem cells, Clinical grade, Cell isolation, Proliferation, Embryonic stem cells, Multipotential capacity

1 Introduction

Multipotent stem cells are of great interest in regenerative medicine due to their potential to repair damaged or diseased tissues. Within the last decade a great deal of innovation has focused on developing such stem cell-based therapeutic strategies, often coupled with tissue engineering approaches, to generate whole functional organs or repair otherwise irreversibly damaged tissue systems. Realization of these regenerative approaches requires extensive expansion of the stem cell population and the ability to control differentiation of the cells towards a specific lineage.

Pluripotent stem cells, such as embryonic stem cells, have generated significant excitement due to their potential to generate all tissues in the body. Effective application of these cell populations for organ regeneration or repair requires logarithmic cell expansion, which, using current culture protocols, is only established using animal serum- or animal-derived matrix components that will likely require immunosuppression in order to sustain transplantation. In addition to carrying the generalized risk of xenograft-induced hypersensitivity reactions, the composition of animal serum is unknown and varies significantly between batches. Fundamentally, this interferes with the reproducibility of experiments and generates a product that may be contaminated with viruses, mycoplasma, prions, or other pathogenic, toxic, and/or immunogenic agents [1–4]. Because of such safety risks, regulatory authorities discourage or prohibit the use of animal sera or other animal product derivatives for the production of biological products for human use [5]. Moreover, current protocols have not effectively shown homogeneous and controlled differentiation of these pluripotent stem cells towards a restricted/terminal cell population. Incompletely or heterogeneously differentiated pluripotent stem cell populations have demonstrated a propensity to form teratomas when used therapeutically [6]. Postnatal multipotent stem cells offer an alternative to pluripotent stem cells in regenerative medicine: their restricted ability to differentiate narrows their potential scope of application, but also excludes the risk of teratoma formation. Further postnatal stem cells circumvent the ethical concerns associated with embryonic stem cell research and have the potential to be derived from an autologous source, thereby preventing immunoreactivity. A number of adult stem cell niches have been discovered, the most established of which is the bone marrow cavity (hematopoietic and mesenchymal stem cells). More recently additional niches for specific stem cell population have been explored, including, fat tissue (adipose-derived stem cells), hair follicle (epithelial bulge), tooth (dental pulp stem cells, DPSCs), and other areas [7], which can each be used to target therapy of specific tissue derivatives. Similar to the pluripotent stem cells, standard expansion and protocols for these cells still involve use of animal sera [8], such as fetal bovine or horse serums.

For these reasons we developed and tested a chemically defined stem cell culture medium that contains only a small amount (1.25 %) of human serum, which can be obtained autologously to replace animal sera during stem cell expansion. This chapter details how this medium can be combined with the isolation and expansion of human DPSCs to produce a rapidly proliferating cell population that expresses pluripotent and mesenchymal stem cell markers. Cells cultured using this medium demonstrate multilineage differentiation capacity [9] and protocols for osteoblastic, neuronal, myocytic, and hepatocytic differentiation are included.

2 Materials

2.1 Disposable Materials

2.1.1 Glassware

1. Graduate class A cylinders (50 mL to 1 L).
2. Conical flasks (50 mL to 1 L).
3. Beakers (50 mL to 1 L).
4. Cover slips 22×22 mm.

All glassware should be sterilized by washing twice in a dishwasher without soap, then wrapped in aluminum foil, and autoclaved at 120 °C and 15 psi for 20 min (or similar sterilizing cycle on approved autoclave systems).

2.1.2 Sterilizing Solutions and Filters

1. 70 % ethanol in H₂O.
2. 100 % isopropanol.
3. 10 % sodium hypochlorite in deionized water (dH₂O).

Culture media and cell culture solutions should be sterilized by filtration through 0.22 µm filters (125 mL to 1 L). Analytical grade reagents are filtered in 0.45 µm filters (125 mL to 1 L).

2.1.3 Laboratory and Disposable Equipment

1. Latex or nitrile gloves.
2. Pointed tipped tissue forceps.
3. 26G×½ in syringe needle; pipette aid; individually wrapped sterile pipettes 1–25 mL.
4. Cell culture-treated petri culture dishes (35–100 mm).
5. Conical bottom tubes (15–50 mL).
6. 6-Multi-well tissue culture-treated plates.
7. Conical bottom sterile microcentrifuge tubes (0.2–1.5 mL).
8. Pipetman (P20, P200, P1000).
9. Disposable sterilized pipette tips (10 µL to 20 µL to 200 µL to 1 mL, tips free of detectable DNA, RNA, DNase, RNase, ATP, pyrogens, and trace metals).
10. Laminar flow cabinet.
11. Heated water bath.
12. Water-jacketed CO₂ incubators for tissue culture.
13. Centrifuge.

2.2 Preparing an Aseptic Environment

All cell culture should be performed in a certified microbiological safety cabinet using aseptic technique to ensure sterility. When the tissue culture hood is not in use, hood sash should remain closed. During use hood sash should be positioned as recommended by the manufacturer to maintain laminar airflow. Use 70 % ethanol to spray all surface areas prior to use and allow to dry under laminar flow. Only sterilized glassware, plastics, pipettes, and pipette tips

should be used for tissue culture. Tissue culture hoods should only contain materials and equipment necessary for the experiment and effort should be made to avoid cluttering the area. Gloves should be worn at all times and sprayed frequently with 70 % ethanol to help maintain sterility. Sterilize forceps using an autoclave, or in a sterile container with 100 % isopropanol for about 5 min, and then let dry in tissue culture hood.

2.3 Culture Media and Reagents

Prepare all media and cell culture solutions using ultrapure double-distilled water (ddH₂O) and analytical grade reagents using distilled water (dH₂O). Store all reagents at 4 °C temperature (unless indicated otherwise). Collect 200 mL human blood serum type O (HS) from consented donors; serum should be screened as negative for hepatitis B, hepatitis C, and human immunodeficiency virus (HIV). Liquid-form media can be purchased from the manufacturer, but should be stored for less than 1 year with minimal exposure to light. Alternatively, to maximize medium performance, media can be made from premixed powder solutions just prior to use. To dissolve powder medium measure out 800 mL of ddH₂O (temperature should be 15–20 °C) in clean 1 L graduated cylinders. While gently stirring the water, add the powdered medium. Stir until dissolved (*see Note 1*). Rinse original package with a small amount of water to remove all traces of powder. Add sodium bicarbonate or sodium bicarbonate solution for each liter of final volume of medium being prepared as requested by the manufacturer, and stir until dissolved. Add additional ddH₂O water to bring the solution to final 1 L volume. Filter sterilize medium using a 0.22 µm filter under a laminar flow tissue culture hood and dispense medium into a sterile container.

1. Fetal bovine serum (FBS), lot specific or stem cell verified: Under laminar flow cabinet aliquot sera into 50 mL conical tubes and store at –20 °C for less than 1 year, or –80 °C for long-term storage. Thaw aliquots of human or bovine serum in heated water bath at 37 °C just prior to mixing with tissue culture media.
2. Under laminar flow cabinet, dilute growth factors, cytokines, and vitamins as suggested in Table 1; dispense in 50–100 µL aliquots; and store at –80 °C.
3. Culture medium F-12 Coon's and Ambesi's modified medium (*see Note 2*).
4. Platelet-derived growth factor-ββ (PDGF-ββ).
5. Epidermal growth factor (EGF).
6. Insulin-like growth factor-1 (IGF-1).
7. Basic fibroblast growth factor (FGF-b, FGF-2).
8. Fibroblast growth factor-4 (FGF-4).
9. Vascular endothelial growth factor (VEGF).

Table 1
Growth factors, cytokines, and vitamins

Media components	Quantity	Conc of stock	Diluted in
Platelet-derived growth factor- $\beta\beta$ (PDGF- $\beta\beta$)	10 μg	10 $\mu\text{g/mL}$	Sterile basal culture media
Epidermal growth factor (EGF)	100 μg	100 $\mu\text{g/mL}$	Sterile basal culture media
Insulin-like growth factor-I (IGF-I)	100 μg	100 $\mu\text{g/mL}$	Sterile basal culture media
Fibroblast growth factor-b (FGF-b)	100 μg	100 $\mu\text{g/mL}$	Sterile basal culture media
Dexamethasone (DEX)	1 mg	0.1 mg/mL	Sterile basal culture media
Ascorbic acid	100 g	100 g/L	Sterile basal culture media
β -Glycerophosphate	10 g	216.04 g/L	Sterile basal culture media
CaCl_2	500 g	110.98 g/L	ddH ₂ O
MgCl_2	500 g	95.21 g/L	ddH ₂ O
Glucose	500 g	200 g/L	ddH ₂ O
Calcitonin	500 μg	500 $\mu\text{g/mL}$	Sterile basal culture media
Vascular endothelial growth factor (VEGF)	10 μg	10 $\mu\text{g/mL}$	Sterile basal culture media
Bone morphogenetic protein-2 (BMP-2)	10 μg	10 $\mu\text{g/mL}$	Sterile basal culture media
Neurotrophin-3 (NT-3)	10 μg	10 $\mu\text{g/mL}$	Sterile basal culture media
Nerve growth factor (NGF)	10 μg	10 $\mu\text{g/mL}$	Sterile basal culture media
Brain-derived neurotrophic factor (BDNF)	10 μg	10 $\mu\text{g/mL}$	Sterile basal culture media
Hepatocyte growth factor (HGF)	10 μg	10 $\mu\text{g/mL}$	Sterile basal culture media
Fibroblast growth factor-4 (FGF-4)	25 μg	25 $\mu\text{g/mL}$	Sterile basal culture media
Insulin	100 mg	100 mg/mL	Sterile basal culture media
Oncostatin	10 μg	10 $\mu\text{g/mL}$	Sterile basal culture media
Nicotinamide	100 g	50 mg/mL	Sterile basal culture media
Low-density lipoprotein (LDL)	5 mg	5 mg/mL	Sterile basal culture media
17 β -Estrogen water soluble	100 mg	25 mg/mL	Sterile basal culture media
Linoleic acid water soluble	1 g	50 mg/mL	Sterile basal culture media
B27 supplement	10 mL		Sterile basal culture media
Gentamicin	50 g/L	50 mg/mL	ddH ₂ O
Vitamin K2	250 mg	50 mg/mL	Chloroform
1,25-Dihydroxyvitamin D3	10 μg	100 $\mu\text{g/mL}$	Ethanol
Butylated hydroxyanisole	5 g	50 mg/mL	Ethanol
3-Isobutyl-1-methylxanthine (IBMX)	100 mg	10 mg/mL	Ethanol
Progesterone	1 mg	50 mg/mL	Chloroform
Indomethacin	5 g	20 mg/ml	Ethanol
Retinoic acid (RA)	50 mg	40 mg/mL	Chloroform

10. B27 supplement.
11. Neurotrophin-3 (NT-3).
12. Nerve growth factor (NGF).
13. Brain-derived neurotrophic factor (BDNF).
14. Hepatocyte growth factor (HGF).
15. Bone morphogenetic protein-2 (BMP-2).
16. Butylated hydroxyanisole.
17. 3-Isobutyl-1-methylxanthine (IBMX).
18. Dexamethasone (DEX).
19. Progesterone.
20. 17- β -estrogen.
21. Vitamin K2.
22. Vitamin D3.
23. Calcitonin.
24. Retinoic acid (RA).
25. Linoleic acid.
26. Oncostatin.
27. Nicotinamide.
28. Low-density lipoprotein (LDL).
29. Indomethacin.
30. Ascorbic acid.
31. Gentamicin.
32. Glucose.
33. β -Glycerophosphate.
34. Collagenase II.
35. Trypsin.
36. Chicken serum.
37. Medium-199.
38. CMRL-1066.
39. DMEM 4.5 g/L glucose.
40. DMEM 1 g/L glucose.
41. Hanks' stock IV solution (20 $\times$).
In 1 L beaker add 800 mL of ddH₂O and weight out 160 g/L NaCl, 7 g/L KCl, 4.97 g/L NaH₂PO₄·7H₂O, and 1.2 g/L KH₂PO₄. Mix by stirring. Add ddH₂O to bring the solution to final 1 L volume, sterile filter, and store at 4 °C.

Table 2**Basal isolation/proliferation culture media with different HS percentages**

Media components	Final conc in media
F-12 Coon's and Ambesi's modified	1/3
Medium-199	1/3
CMRL-1066	1/3
Human blood serum type O (HS) ^a	1.25 or 2.5 or 0.5 or 0.25 %

^aFrom consented donors

42. Basal isolation/proliferation culture media with different HS percentages.

Under laminar flow cabinet, measure an equal volume for all of each F-12 Coon's and Ambesi's modified, Medium-199 and CMRL-1066 media in a graduated cylinder obtaining basal culture media. Thaw at 37 °C in heated water bath a 50 mL conical tube filled with HS and add 1.25, 2.5, 0.5, or 0.25 % human serum in the 1 L sterile graduated cylinder (Table 2). While stirring, adjust the pH of the medium to 0.1–0.3 pH units below the desired pH (7.2) since it may rise during filtration. Add additional basal isolation/proliferation culture medium to bring the solution to final 1 L volume. Solution should be filter sterilized and stored at 4 °C.

43. Complete isolation/proliferation culture medium.

Under laminar flow cabinet, in 90 mL basal isolation/proliferation medium with 1.25 % HS add 25 ng/mL PDGF-ββ, 25 ng/mL EGF, 25 ng/mL IGF-1, 25 ng/mL FGF-b, 3.9 ng/mL DEX, 90 μg/L linoleic acid, 25 mg/L ascorbic acid, and 25 μg/mL gentamicin (Table 3). While stirring, adjust the pH of the medium to 6.9–7.1 (the final pH should be 7.2, but pH may rise during filtration) using sterile 1 N HCl or 1 N NaOH. Finally add, under laminar flow cabinet, required volume of basal isolation/proliferation medium with 1.25 % HS content to bring the solution to final 100 mL volume. Filter sterilize the complete isolation/proliferation culture medium and store for a maximum of 2 weeks at 4 °C (*see Note 3*).

44. Osteoblast differentiation medium.

Under laminar flow cabinet, measure out about 80 % of final required volume of F-12 Coon's and Ambesi's modified and transfer in 100 mL clean graduated cylinder. To medium add 0.5 % HS, 133.17 mg/L CaCl₂, 57.12 mg/L MgCl₂, 2 g/L glucose, 216.04 mg/L β-glycerophosphate, 75.11 ng/mL RA, 39.24 ng/mL DEX, 275.4 μg/mL 17-β-estrogen, 444.65 μg/mL vitamin K2, 2.08 ng/mL vitamin D3, 3.43 ng/mL calcitonin, 15 ng/mL BMP-2, and 50 μg/mL gentamicin (Table 4). While stirring, adjust the pH of the medium to 6.9–7.1 using

Table 3

Complete isolation/proliferation culture medium added with 1.25 % human serum and complete isolation/proliferation culture medium added with different human serum percentages

Media components	Conc of stock	Diluted in	Final conc in media
Basal culture media with different HS percentages			~100 mL
Platelet-derived growth factor- $\beta\beta$ (PDGF- $\beta\beta$)	10 $\mu\text{g/mL}$	Sterile basal culture media	25 ng/mL
Epidermal growth factor (EGF)	100 $\mu\text{g/mL}$	Sterile basal culture media	25 ng/mL
Insulin-like growth factor-I (IGF-I)	100 $\mu\text{g/mL}$	Sterile basal culture media	25 ng/mL
Fibroblast growth factor-b (FGF-b)	100 $\mu\text{g/mL}$	Sterile basal culture media	25 ng/mL
Dexamethasone (DEX)	0.1 mg/mL	Sterile basal culture media	3.9246 ng/mL
Ascorbic acid	100 g/L	Sterile basal culture media	25 mg/L
Linoleic acid water soluble	50 mg/mL	Sterile basal culture media	90 $\mu\text{g/L}$
Gentamicin	50 mg/mL	ddH ₂ O	25 $\mu\text{g/mL}$

Table 4

Osteoblast differentiation medium

Media components	Conc of stock	Diluted in	Final conc in media
F-12 Coon's and Ambesi's modified			~100 mL
Human blood serum type O (HS)			0.5 %
CaCl ₂	110.98 g/L	ddH ₂ O	133.17 mg/L
MgCl ₂	95.21 g/L	ddH ₂ O	57.12 mg/L
Glucose	200 g/L	ddH ₂ O	2 g/L
β -Glycerophosphate	216.04 g/L	Sterile basal culture media	216.04 mg/L
Retinoic acid (RA)	40 mg/mL	Chloroform	75.11 ng/mL
Dexamethasone (DEX)	0.1 mg/mL	Sterile basal culture media	39.246 ng/mL
17 β -Estrogen water soluble	25 mg/mL	Sterile basal culture media	275.4 $\mu\text{g/mL}$
Vitamin K2	50 mg/mL	Chloroform	444.65 $\mu\text{g/mL}$
1,25-Dihydroxyvitamin D3	100 $\mu\text{g/mL}$	Ethanol	2.083 ng/mL
Calcitonin	500 $\mu\text{g/mL}$	Sterile basal culture media	3.43185 ng/mL
Bone morphogenetic protein-2 (BMP-2)	10 $\mu\text{g/mL}$	Sterile basal culture media	15 ng/mL
Gentamicin	50 mg/mL	ddH ₂ O	50 $\mu\text{g/mL}$

Table 5
Myocyte differentiation medium

Media components	Conc of stock	Diluted in	Final conc in media
DMEM 1 g/L glucose			~ 100 mL
Fetal bovine serum (FBS)			5 %
Fibroblast growth factor-b (FGF-b)	100 µg/mL	Sterile basal culture media	10 ng/ml
Vascular endothelial growth factor (VEGF)	10 µg/mL	Sterile basal culture media	10 ng/mL
Insulin-like growth factor-I (IGF-I)	100 µg/mL	Sterile basal culture media	10 ng/ml
Gentamicin	50 mg/mL	ddH ₂ O	50 µg/mL

sterile 1 N HCl or 1 N NaOH. Finally add, under laminar flow cabinet, required volume of F-12 Coon's and Ambesi's modified to bring the solution to final 100 mL volume. Filter sterilize the osteoblast differentiation medium and store for a maximum of 2 weeks at 4 °C.

45. Myocyte differentiation medium.

Under laminar flow cabinet, measure out about 80 % of final required volume of DMEM 1 g/L glucose and transfer to a sterile 100 mL graduated cylinder. To low-glucose DMEM add 5 % FBS serum, 10 ng/mL FGF-b, 10 ng/mL VEGF, 10 ng/mL IGF-1, and 50 µg/mL gentamicin (Table 5). While stirring, adjust the pH of the medium to 0.1–0.3 pH units below the desired pH (7.2) since it may rise during filtration. The use of 1 N HCl or 1 N NaOH is recommended. Finally add, under laminar flow cabinet, required volume of DMEM 1 g/L glucose to bring the solution to final 100 mL volume. Sterilize immediately by filtration the myocyte differentiation medium and dispense medium into sterile container. Close bottle with cap maintaining sterility and store at 4 °C.

46. Neural specification medium.

Under laminar flow cabinet, measure out about 80 % of final required volume of DMEM 4.5 g/L glucose and transfer in a 100 mL clean graduated cylinder. Thaw at 37 °C in heated water bath a 50 mL conical tube filled with FBS and add 10 % serum and gentamicin 50 µg/mL (Table 6). Finally add, under laminar flow cabinet, required volume of DMEM 4.5 g/L glucose to bring the solution to final 100 mL volume. Filter sterilize the neural specification medium and store for a maximum of 2 weeks at 4 °C.

47. Neural commitment medium.

Under laminar flow cabinet, measure out about 80 % of final required volume of DMEM 4.5 g/L glucose and transfer to

Table 6
Neural differentiation media

Media components	Conc of stock	Diluted in	Final conc in media
<i>Neural specification medium</i>			
DMEM 4.5 g/L glucose			~100 mL
Fetal bovine serum (FBS)			10 %
Gentamicin	50 mg/mL	ddH ₂ O	50 µg/mL
<i>Neural commitment medium</i>			
DMEM 4.5 g/L glucose			~100 mL
Fetal bovine serum (FBS)			10 %
B27 supplement		Sterile basal culture media	5 mL/L
Fibroblast growth factor-b (FGF-b)	100 µg/mL	Sterile basal culture media	20 ng/mL
Epidermal growth factor (EGF)	100 µg/mL	Sterile basal culture media	10 ng/mL
Gentamicin	50 mg/mL	ddH ₂ O	50 µg/mL
<i>Second neural commitment medium</i>			
DMEM 4.5 g/L glucose			~100 mL
Fetal bovine serum (FBS)			5 %
B27 supplement		Sterile basal culture media	5 mL/L
Epidermal growth factor (EGF)	100 µg/mL	Sterile basal culture media	5 ng/mL
Fibroblast growth factor-b (FGF-b)	100 µg/mL	Sterile basal culture media	10 ng/mL
Neurotrophin-3 (NT-3)	10 µg/mL	Sterile basal culture media	10 ng/mL
Nerve growth factor (NGF)	10 µg/mL	Sterile basal culture media	10 ng/mL
Brain-derived neurotrophic factor (BDNF)	10 µg/mL	Sterile basal culture media	25 ng/mL
Butylated hydroxyanisole	50 mg/mL	Ethanol	1.802 mg/mL
3-Isobutyl-1-methylxanthine (IBMX)	10 mg/mL	Ethanol	5.556 mg/mL
Retinoic acid (RA)	40 mg/mL	Chloroform	150.22 µg/mL
Progesterone	50 mg/mL	Chloroform	3.1446 ng/mL
Gentamicin	50 mg/mL	ddH ₂ O	50 µg/mL
<i>Neural final differentiation medium</i>			
DMEM 4.5 g/L glucose			~100 mL
Brain-derived neurotrophic factor (BDNF)	10 µg/mL	Sterile basal culture media	50 ng/mL
Insulin	100 mg/mL	Sterile basal culture media	5 µg/mL
Indomethacin	20 mg/mL	Ethanol	71.558 ng/mL
3-Isobutyl-1-methylxanthine (IBMX)	10 mg/mL	Ethanol	111.12 µg/mL
Gentamicin	50 mg/mL	ddH ₂ O	50 µg/mL

100 mL clean graduated cylinder. To high-glucose DMEM add 10 % FBS, 5 mL/L B27 supplement, 10 ng/mL EGF, 20 ng/mL FGF-b, and 50 µg/mL gentamicin (Table 6). While stirring, adjust the pH of the medium to 6.9–7.1 using sterile 1 N HCl or 1 N NaOH. Finally add, under laminar flow cabinet, required volume of DMEM 4.5 g/L glucose to bring the solution to final 100 mL volume. Filter sterilize the neural commitment medium and store for a maximum of 2 weeks at 4 °C.

48. Secondary neural commitment medium.

Under laminar flow cabinet, measure out about 80 % of final required volume of DMEM 4.5 g/L glucose and transfer to a

100 mL clean graduated cylinder. To high-glucose DMEM add 5 % FBS, 5 mL/L B27, 5 ng/mL EGF, 10 ng/mL FGF-b, 10 ng/mL NT-3, 10 ng/mL NGF, 25 ng/mL BDNF, 1.8 mg/mL butylated hydroxyanisole, 5.5 mg/mL IBMX, 150.22 µg/mL RA, 3.14 ng/mL progesterone, and 50 µg/mL gentamicin (Table 6). While stirring, adjust the pH of the medium to 6.9–7.1 using sterile 1 N HCl or 1 N NaOH. Finally add, under laminar flow cabinet, required volume of DMEM 4.5 g/L glucose to bring the solution to final 100 mL volume. Filter sterilize the secondary neural commitment medium and store for a maximum of 2 weeks at 4 °C.

49. Final neural differentiation medium.

Under laminar flow cabinet, measure out about 80 % of final required volume of DMEM 4.5 g/L glucose and transfer to 100 mL clean graduated cylinder. To high-glucose DMEM add 50 ng/mL BDNF, 5 µg/mL insulin, 71.5 ng/mL indomethacin, 111.12 µg/mL IBMX, and 50 µg/mL gentamicin (Table 6). While stirring, adjust the pH of the medium to 6.9–7.1 using sterile 1 N HCl or 1 N NaOH. Finally add, under laminar flow cabinet, required volume of DMEM 4.5 g/L glucose to bring the solution to final 100 mL volume. Filter sterilize the final neural differentiation medium and store for a maximum of 2 weeks at 4 °C.

50. Hepatocyte differentiation medium.

Under laminar flow cabinet, measure about 80 % of final required volume of DMEM 1 g/L glucose and transfer to a 100 mL clean graduated cylinder. To low-glucose DMEM add 1 % FBS, 20 ng/mL HGF, 10 ng/mL oncostatin, 1.22 mg/mL nicotinamide, 1.25 µg/mL LDL, 10 ng/mL FGF-4, 4 µg/mL insulin, 180 µg/mL linoleic acid, and 50 µg/mL gentamicin (Table 7). While stirring, adjust the pH of the medium to 6.9–7.1 using sterile 1 N HCl or 1 N NaOH. Finally add, under laminar flow cabinet, required volume of DMEM 1 g/L glucose to bring the solution to final 100 mL volume. Filter sterilize the final hepatocyte medium and store for a maximum of 2 weeks at 4 °C.

51. Detaching solution (CTC).

Completely submerge a 1 L beaker in ice and add in the following order: 600 mL ddH₂O, 0.5 U/mL trypsin (final concentration), 20 mL dialyzed and heat-inactivated chicken serum, 1.27 g/L NaHCO₃, 2 g/L glucose, 50 mL 20× Hanks' stock IV solution, and 22 U/mL collagenase II. Mix components in chilled beaker with gentle stirring. Transfer solution to a graduated cylinder and add ddH₂O to bring the solution to a final volume of 1 L. Sterilize immediately by filtration and aseptically dispense solution into sterile 50 mL tubes for long-term storage at –80 °C.

Table 7
Hepatocyte differentiation medium

Media components	Conc of stock	Diluted in	Final conc in media
DMEM 1 g/L glucose			~100 mL
Fetal bovine serum (FBS)			1 %
Hepatocyte growth factor (HGF)	10 µg/mL	Sterile basal culture media	20 ng/mL
Oncostatin	10 µg/mL	Sterile basal culture media	10 ng/mL
Nicotinamide	50 mg/mL	Sterile basal culture media	1.221 mg/mL
Low-density lipoprotein (LDL)	5 mg/mL	Sterile basal culture media	1.25 µg/mL
Fibroblast growth factor-4 (FGF-4)	25 µg/mL	Sterile basal culture media	10 ng/mL
Insulin	100 mg/mL	Sterile basal culture media	4 µg/mL
Linoleic acid water soluble	50 mg/mL	Sterile basal culture media	90 µg/L
Gentamicin	50 mg/mL	ddH ₂ O	50 µg/mL

2.4 Agar-Covered Dishes for DPSC Osteoblastic Induction

Weigh out 4 g of agarose into a 500 mL beaker with 100 mL ddH₂O, and swirl to mix. Microwave for about 2 min to dissolve the agarose. Transfer agar to 500 mL bottle (keep cap loose) and autoclave to sterilize. Under sterile conditions, gently mix a 1:1 vol/vol ratio of hot 4 % agar solution and osteoblast differentiation medium. Dispense 7 mL of this solution per 100 mm petri dish, and rock back and forth to make sure that dish is evenly coated. Wait until solution has cooled and cover with petri lid. Plates can be stored for up to 2 weeks at 4 °C (*see Note 4*).

2.5 Growth Curves

1. Phosphate-buffered saline (PBS 10×) pH 7.4.
In 1 L beaker add 800 mL ddH₂O, 80 g/L NaCl, 2 g/L KCl, 14.4 g/L Na₂HPO₄, and 2.4 g/L KH₂PO₄. Mix by stirring. Add ddH₂O to bring the solution to final 1 L volume, sterile filter, and store at 4 °C.
2. 0.4 % Trypan blue.
Add 4 g/L trypan blue to 100 mL 1× PBS, mix by stirring, and store at 4 °C.
3. Neubauer chamber.

2.6 Cell Cryopreservation

1. Cryovials (1.5–2 mL).
2. Freezing container.
3. DMSO.

3 Methods

3.1 Dental Pulp Isolation

1. Immediately after tooth is lost, preserve specimen in a sterile container at 4 °C until ready to perform primary cell culture (*see Note 5*). Alternatively teeth can be conserved and transported in a container filled with freshly opened pasteurized milk to exploit milk's antimicrobial properties (Fig. 1a).
2. Under sterilized laminar flow cabinet hold teeth using sterilized forceps and extract dental pulp using a 26G $\times \frac{1}{2}$ in. syringe

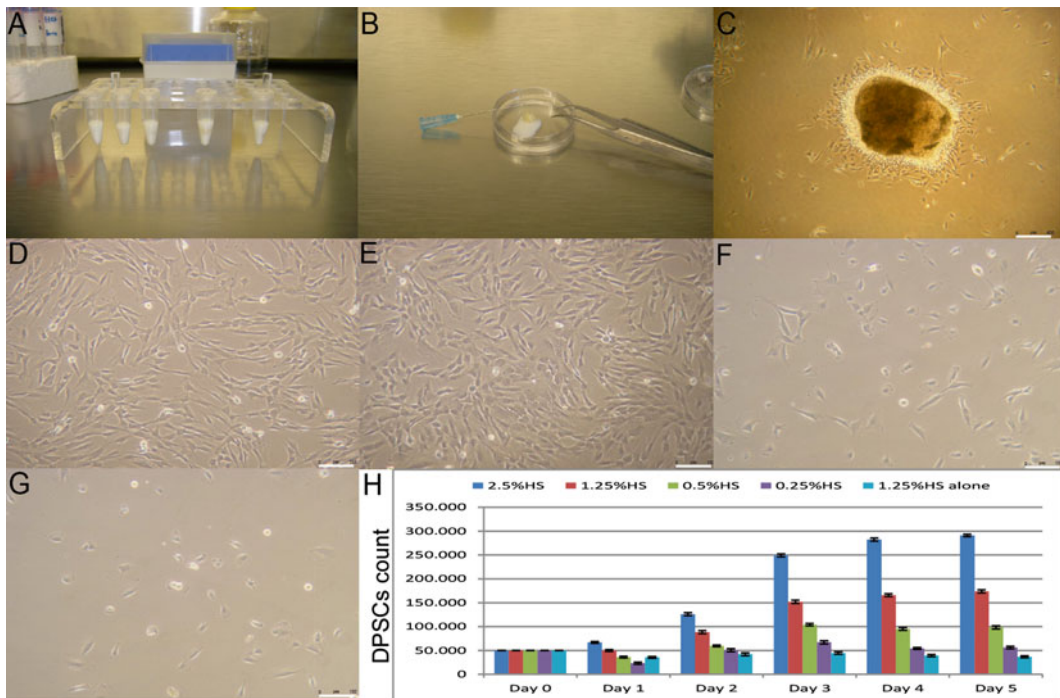


Fig. 1 DPSC morphology and growth curves. (a) Teeth conserved in milk in 1.5 mL conical tube. (b) Primary dental pulp cell extraction beginning. (c) After 5 days of culture, a robust exodus of fibroblast-like cells from the dental pulp fragments is observed, bar scale 400 μ m. (d) After 2 weeks in complete isolation/proliferation culture medium added with 1.25 % HS, isolated DPSCs are morphologically uniform and highly proliferative, with reduced cytoplasm, bar scales 150 μ m. DPSC morphology in complete isolation/proliferation culture medium, added with 2.5 % HS (e), 0.5 % HS medium (f), or 0.25 % HS medium (g). (h) 5×10^4 DPSCs plated in 60 mm well, in the presence of complete isolation/proliferation culture medium, added with 2.5 % HS or 1.25 % HS or 0.5 % HS or 0.25 % HS, and basal isolation/proliferation culture medium added with 1.25 % HS. Estimated doubling time, expressed as mean $\pm$ SD, for complete isolation/proliferation culture medium added with 1.25 % HS 2.5 and 1.25 % HS was 25.3 ± 1.5 h and 29.8 ± 1.3 h, respectively. Doubling time for cells in media with 0.5 % and 0.25 % and basal isolation/proliferation culture medium added with 1.25 % HS was 31.4 ± 1.2 , 31.4 ± 2 , and 146 ± 1 h, respectively. X-DPSC count Y-medium type ($p < 0.05$). Images from Ferro F, Spelat R, Beltrami AP, Cesselli D, Curcio F. (2012) Isolation and characterization of human dental pulp derived stem cells by using media containing low human serum percentage as clinical grade substitutes for bovine serum. Reproduced from [32]

needle to scrape the pulp tissue out of the pulpal cavity (*see Note 6*) (Fig. 1b); usually extraction results in some fragmentation of the dental pulp.

3. Transfer dental pulp fragments into 35 mm petri culture dish in the presence of 1.5 mL (*see Note 7*) complete isolation/proliferation culture medium supplemented with 1.25 % HS (Table 3) and pre-warmed to 37 °C (*see Note 8*).
4. Repeat pulp extraction into a second 35 mm culture dish to ensure that the tooth pulpal cavity is completely empty and separate the cultures in case of contamination (*see Note 9*). Transfer to incubator in humidified atmosphere of 95 % air and 5 % CO₂ at 37 °C and do not move for at least 3 days (*see Note 10*).
5. After 3 days in incubator check culture under microscope to ensure that pulp tissue is healthy and cells have started to outgrow onto tissue culture plastic. The gradient between the piece of dental pulp and the culture medium serves as a vector directing the cells towards what they perceive as a site of injury, which leads to their continued and selective migration in the culture dish [9, 10]. Healthy cultures should cause the color of the medium to change from pinky/orange to orange/yellow as the cells metabolize media components and change the pH.

3.2 Medium Change

1. Three days after the start of the DPSC primary culture, aspirate the old culture medium and immediately replace with 2.5 mL of pre-warmed complete isolation/proliferation culture medium (*see Note 11*). Old medium should be treated as biohazardous waste as recommended by your facility. Media should be changed twice a week. Cells should be attached to the bottom of the culture dish and continue to migrate out from dental pulp pieces with round and plump or elongated shape and refracting light around their membrane (Fig. 1c) (*see Note 12*).
2. DPSC colonies should develop in primary culture after about 1 week and reach confluence in about 2–3 weeks. To compare DPSC expansion at different serum concentrations DPSCs should be isolated in the same manner as described, but the HS component of the complete isolation/proliferation culture medium can be changed from 0.25 to 0.5–2.5 % (Fig. 1d–g).

3.3 Cell Passage

1. Aspirate old culture medium from the primary DPSC culture plates (passage 0, “P0”) and transfer it to a 15 mL sterile conical tube. Immediately add cell-detaching solution (0.75 mL CTC per 35 mm culture dish) (*see Notes 11 and 13*). Place culture dish in incubator at 37 °C for 2–5 min until all cells have detached. Detached cells should look round and plump

and refract light around their membrane; some cells may clump (this is fine).

2. Add old culture medium to plate to inhibit CTC and then transfer cell solution to 15 ml conical tube. Centrifuge at $1,000 \times g$ for 5 min. Aspirate medium from the cell pellet at the bottom of the 15 mL conical tube being careful not to disrupt cell pellet.
3. Resuspend cell pellet in new, pre-warmed, complete isolation/proliferation culture medium by gently pipetting up and down to obtain single-cell suspension (*see Note 14*). Bring passaged cells to a volume of 7 mL media and transfer solution to 100 mm culture dish at passage 1 (P1) (*see Notes 15 and 16*).
4. Once cells reach ~70–80 % confluence (3 days, Fig. 1d), repeat cell passaging protocol just described and plate 2×10^3 cells/cm² onto 100 mm dishes (*see Note 17*). Change medium every 3 days.

3.4 Growth Curves

1. Once DPSC culture has been expanded to passage 4 (P4) detach, as previously indicated, DPSC isolated and cultured in complete isolation/proliferation culture medium, as well as in media containing different HS percentages, from culture dish and plate at P5, in triplicate, 5×10^4 cells in 60 mm culture dishes.
2. Count cells every day from day 1 to day 5, without medium changing. Detach and resuspend cells as previously described in 2 mL medium “resuspension volume” (*see Note 18*).
3. Take 200 μ L of cell suspension and add 20 μ L of 0.4 % trypan blue solution, to highlight nonviable cells. Put the 22×22 mm cover slip on the Neubauer chamber central area. Adjust the micropipette to suck 10 μ L, and load micropipette with 10 μ L of cells in medium plus 0.4 % trypan blue. Place pipette tip close to the coverslip edge, right at the center of the Neubauer chamber. Release the plunger slowly watching how the liquid enters the chamber uniformly, being absorbed by capillarity. Place the Neubauer chamber on the microscope stage. Count cells in the four-grid square, write the amount of cells counted for all single square, and repeat count in triplicate for all samples. Obtain cell concentration using the general formula

$$\text{Concentration (cell / mL)} = \frac{\text{Number of cells}}{\text{Volume (in mL)}}$$

For the Neubauer chamber, the formula used when counting in the big squares is

$$\text{Concentration} = \frac{\text{Number of cells} \times 10.000}{\text{Number of squares}}$$

$$\text{Total cell number} = \frac{\text{Number of cells} \times 10.000}{\text{Number of squares}} \times \text{Total resuspension volume}$$

In case a dilution was applied, the concentration obtained should be converted to the original concentration before the dilution. In this case, the concentration should be divided by the dilution applied. The formula will be

$$\text{Concentration} = \frac{\text{Number of cells} \times 10.000}{\text{Number of squares} \times \text{dilution}} \times \text{Total resuspension volume}$$

$$\text{Total cell number} = \frac{\text{Number of cells} \times 10.000}{\text{Number of squares} \times \text{dilution}}$$

3.5 Population Doubling Time

Calculate population doubling time during logarithmic growth phase by using doubling time software v1.0.10 (<http://www.doubling-time.com>) [11] (Fig. 1h).

The number of total cell generations could be obtained by dividing total culture time, expressed in hours, for the doubling time. Cell doubling exponential rate and the number of total generations will serve us to evaluate the number of total cells starting from two progenitor cells. Then total cells, calculated from data obtained from P0 to P5, should be divided by the number of total cells starting from two progenitor cells, estimating approximately the number of the primary culture progenitor cells.

3.6 Cell Cryopreservation

1. Cryopreservation of DPSC should be done on 70–80 % confluent plates and absence of bacterial or fungal contamination should be confirmed under the microscope (*see* **Note 19**).
2. Remove and store old medium in a 15 mL sterile conical tube. Wash cells with 5 mL 1× PBS sterile and detach cells using 2 mL of CTC solution per 100 mm culture dish. Harvest cells by diluting CTC solution with old medium.
3. Centrifuge at 1,000×*g* for 5 min, discard supernatant, and resuspend cells with freezing medium (complete isolation/proliferation medium supplemented with 1.25 % HS) (Table 3) with 10 % DMSO at a concentration of 2 × 10⁶ per mL.
4. Add 1 mL of cells into labeled 1.5 mL cryovials. Place cryovials on ice for 1 h before transferring to a freezing container filled with isopropanol and placing in the −80 °C freezer overnight. The appropriate rate of cooling is 2 °C per minute. Transfer the vials to liquid nitrogen for long-term storage.

3.7 DPSC Characterization and Differentiation

Once obtained a discrete number of cells, approximately 60 × 10⁶ total cells at P5, harvest and/or collect samples to perform analysis, such as immunofluorescence, fluorescence-activated cell sorting,

PCR, real time-PCR, immunoblot, and multilineage differentiation [9, 12–21] (Fig. 2).

3.8 Multilineage Potential

Confirm multipotency differentiating DPSC towards osteoblastic, hepatic, myocytic, and neural lineages as follows, in a 5 % CO₂, 95 % humidity incubator at 37 °C with medium change twice a week, and then subject differentiated cells to characterization.

3.9 Two-Dimensional (2D) Bidimensional Osteoblast Culture

1. Isolate, detach, count, and plate P5 DPSCs at a concentration of 4×10^4 cells/cm² onto a 100 mm culture dish (i.e., for western blot analysis and PCR) and 6× culture multi-well plates, previously covered with sterile cover slip 22×22 mm (i.e., for immunohistochemistry or immunofluorescence staining) (*see Note 20*), in, respectively, 7 mL and 2 mL osteoblast differentiation medium.
2. DPSCs should be maintained in the osteoblast differentiation medium (Table 4) for 1 month with media changed twice per week (*see Note 21*). Once differentiated harvest and/or collect samples to perform analysis (i.e., immunofluorescence, PCR, immunoblot, chemical and physical analysis) (Fig. 3).

3.10 Three-Dimensional (3D) Osteoblast Aggregate Culture Condition

1. Isolate, detach, and count 5×10^6 P5 DPSCs. Aggregates are formed by placing 5×10^6 cells into 15 mL conical tubes and centrifuging at $1,000 \times g$ for 5 min.
2. Carefully detach the aggregate from the bottom of the conical tube by cutting the tip off of a sterile 5 mL pipette and gently aspirating the aggregate. Transfer aggregate to a 100 mm petri dish coated in 2 % agarose, cover in 30 mL of osteoblast differentiation medium (Table 4), and culture for 3 months.
3. Do not change the medium on the aggregate during the first week to prevent disturbing the cellular interactions (*see Note 22*).
4. Afterwards medium should be changed twice a week (*see Note 21*). Once differentiated harvest and/or collect samples to perform analysis (i.e., immunofluorescence, PCR, immunoblot, chemical and physical analysis) (Fig. 3).

3.11 Myocyte Differentiation

1. Isolate, detach, count, and plate P5 DPSCs at a concentration of 4×10^4 cells/cm² in 100 mm culture dish (i.e., for western blot analysis and PCR) and 6× culture multi-well plates, previously covered with sterile cover slip 22×22 mm (i.e., for immunohistochemistry or immunofluorescence staining) (*see Note 20*), in, respectively, 7 mL and 2 mL myocyte differentiation medium.
2. DPSCs should be maintained in the myocyte differentiation medium (Table 5) for 1 month with media changed twice per week (*see Note 21*). Once differentiated harvest and/or collect samples to perform analysis (i.e., immunofluorescence, PCR) (Fig. 4).

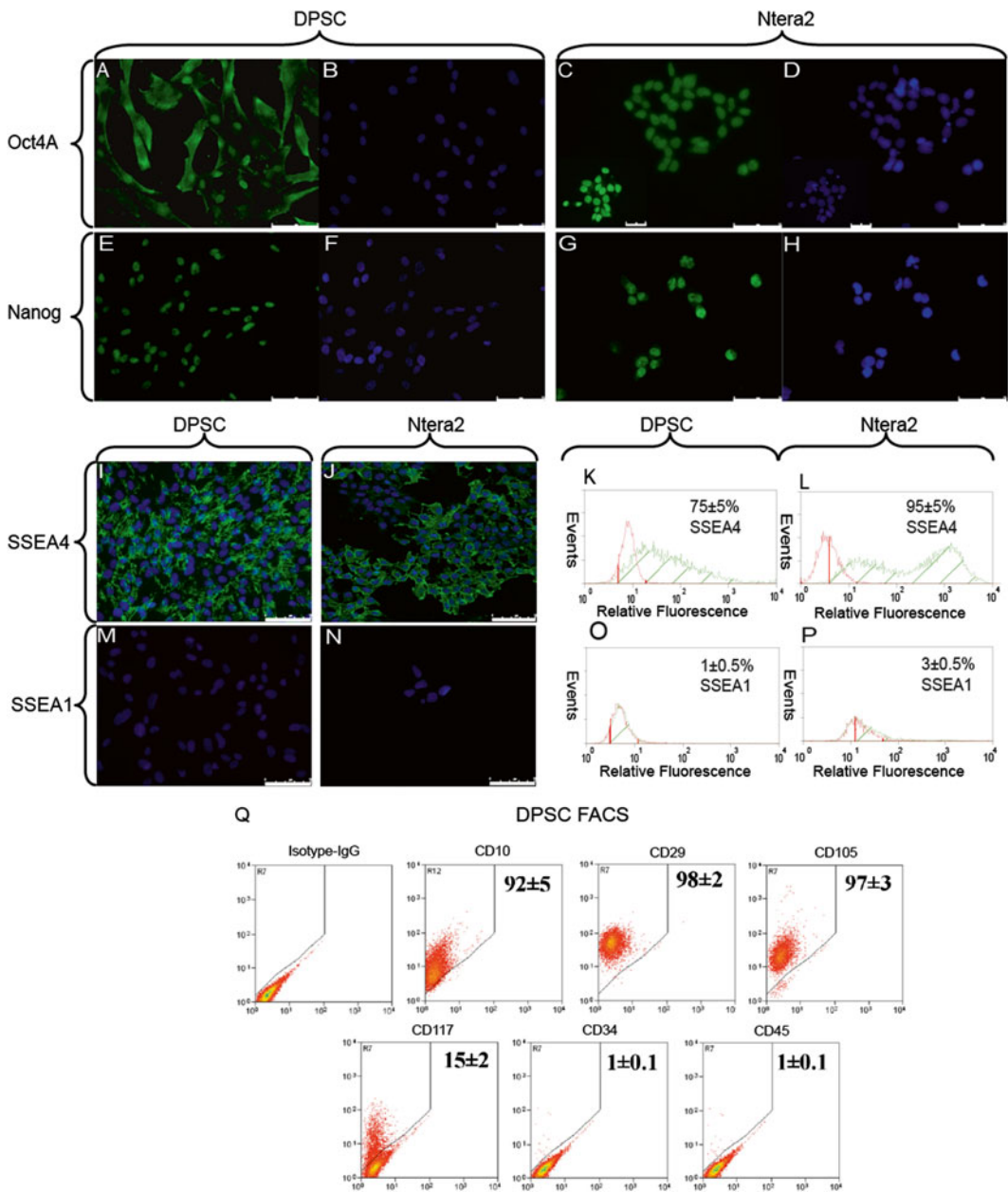


Fig. 2 Embryonic stem cell markers. FITC-labeled antibody was used to evidence Nanog expression in DPSC (a and b) and Ntera2 (c and d) (Ctr +), Oct4A expression in DPSC (e and f) and Ntera2 (g and h). SSEA4 expression in DPSC (i and k) and Ntera2 (j and l) by immunofluorescence and FACS, respectively. TRITC-labeled antibody was used to evaluate, by IF, SSEA1 expression in DPSC (m) and Ntera2 (n) and by FACS DPSC (o) and Ntera2 (p). Scale bars 50–75 μ m. Nuclei were counterstained with DAPI. X-axis shows relative fluorescence and Y-axis the number of events, indicating the percentage of positive cells (mean $\pm$ standard deviation) of $n=3$ experiments (adjusted $P<0.05$). (q) CD representative flow cytometry for dot plots of undifferentiated and differentiated DPSC. Gate was defined considering the isotype control IgG-stained sample versus specific antibody staining profile. Results reported in each plot, X-axis shows relative fluorescence and Y-axis the number of events, indicating the percentage of positive cells (mean $\pm$ standard deviation) of $n=3$ experiments (adjusted $P<0.05$). Reproduced from [32]

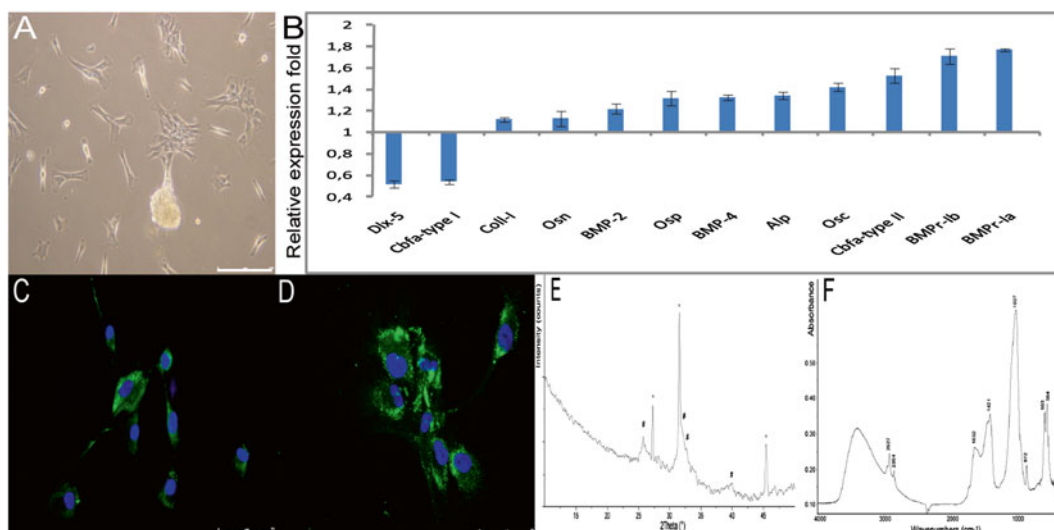


Fig. 3 DPSC osteoblastic differentiation characterization. (a) Evidence of the osteo-differentiated DPSC morphology change, bar scales 150 μm . (b) Semiquantitative rt-PCR was used to determine the gene expression profile ratio of osteoblastic specific markers in osteoblastic differentiated over undifferentiated cells, relative to housekeeping gene. Values were expressed as mean $\pm$ SD and were normalized using β -actin. X-axis shows relative expression fold and Y-axis tested markers (adjusted $P < 0.05$). (c) FITC-labeled antibody was used to evaluate osteocalcin expression in osteoblastic differentiated DPSC, bar scales 75 μm . (d) FITC-labeled antibody was used to evaluate osteopontin expression in osteoblastic differentiated DPSC, bar scales 75 μm . Nuclei were counterstained with DAPI. (e) X-ray powder diffraction patterns from the DPSC aggregates after osteoblastic induction. The main diffraction peaks of carbonate hydroxyapatite (#) and sodium chloride (°) and the wave numbers of the absorption peaks are indicated. (f) The FTIR spectrum shows absorption peaks at the characteristic peaks of phosphate group ($\nu^3 = 1,037 \text{ cm}^{-1}$; $\nu^4 = 603 \text{ cm}^{-1}$ and 561 cm^{-1}) and carbonate group ($\nu^3 = 1,421 \text{ cm}^{-1}$). Reproduced from [32]

3.12 Neural Differentiation

Neural differentiation proceeds in three sequential steps: specification, commitment, and differentiation.

1. Isolate, detach, count, and plate P5 DPSCs at a concentration of 3×10^3 cells/ cm^2 in 100 mm culture dish (i.e., for western blot analysis and PCR) and 6x culture multi-well plates, previously covered with sterile cover slip $22 \times 22 \text{ mm}$ (i.e., for immunohistochemistry or immunofluorescence staining) (see **Note 20**), in, respectively, 7 mL and 2 mL neural specification medium (Table 6) for 24 h.
2. Then cells were shifted to a neural commitment medium (Table 6) and cultured for 15 days.
3. Afterward, cells were cultured for 15 days in a second neural commitment medium (Table 6).
4. Finally, neural differentiation was achieved by exposing committed cells to a neural final differentiation medium (Table 6) for 1 day; cells should be maintained in a 100 % humidified atmosphere of 95 % air and 5 % CO_2 at 37°C with media

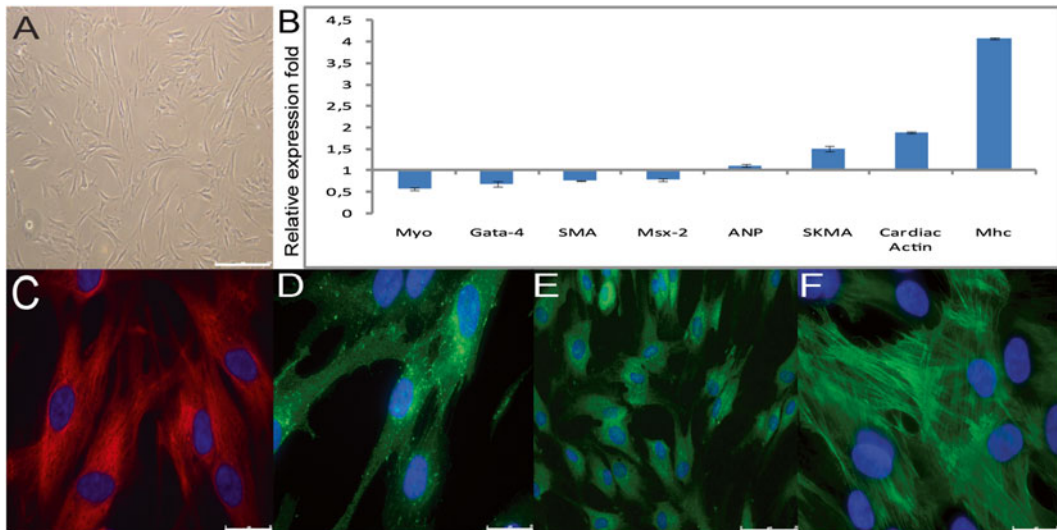


Fig. 4 DPSC myocyte differentiation characterization. (a) Evidence of the myocyte-differentiated DPSC morphology change, bar scales 150 μ m. (b) Semiquantitative rt-PCR was used to determine the gene expression profile ratio of myocyte-specific markers in myocyte-differentiated over undifferentiated DPSC cells. Values were expressed as mean $\pm$ SD and were normalized using β -actin. X-axis shows relative expression fold and Y-axis tested markers (adjusted $P < 0.05$). (c) TRITC-labeled antibody was used to evaluate sarcomeric actin expression in myocyte-differentiated DPSC, bar scales 25 μ m. (d) FITC-labeled antibody was used to evaluate connexin-43 expression in myocyte-differentiated DPSC, bar scales 25 μ m. (e) FITC-labeled antibody was used to evaluate ATPase pump serca 2 expression in myocyte-differentiated DPSC, bar scales 75 μ m. (f) FITC-labeled antibody was used to evaluate smooth muscle actin expression in myocyte-differentiated DPSC, bar scales 25 μ m. Nuclei were counterstained with DAPI. Reproduced from [32]

changed twice per week (*see Note 21*). Once differentiated harvest and/or collect samples to perform analysis (i.e., immunofluorescence, PCR) (Fig 5).

3.13 Hepatocyte Differentiation

1. Hepatocyte differentiation is obtained in confluent DPSC at P5 in hepatocyte medium (Table 7) in 100 mm culture dish (i.e., for western blot analysis and PCR) and 6 $\times$ culture multi-well plates, previously covered with sterile cover slip 22 $\times$ 22 mm (i.e., for immunohistochemistry or immunofluorescence staining) (*see Note 20*), in, respectively, 7 mL and 2 mL.
2. Cells should be maintained in a 100 % humidified atmosphere of 95 % air and 5 % CO₂ at 37 $^{\circ}$ C for 40 days with media changed twice per week (*see Note 21*). Once differentiated harvest and/or collect samples to perform analysis (i.e., immunofluorescence, PCR, enzyme-linked immunosorbant assay) (Fig. 6).

3.14 Cell Line Positive Controls

Human embryonic carcinoma stem cells (Ntera2) can be used as positive control for embryonic stem markers as suggested by Liedtke et al. [22]. MCF7 breast cancer cell line is negative

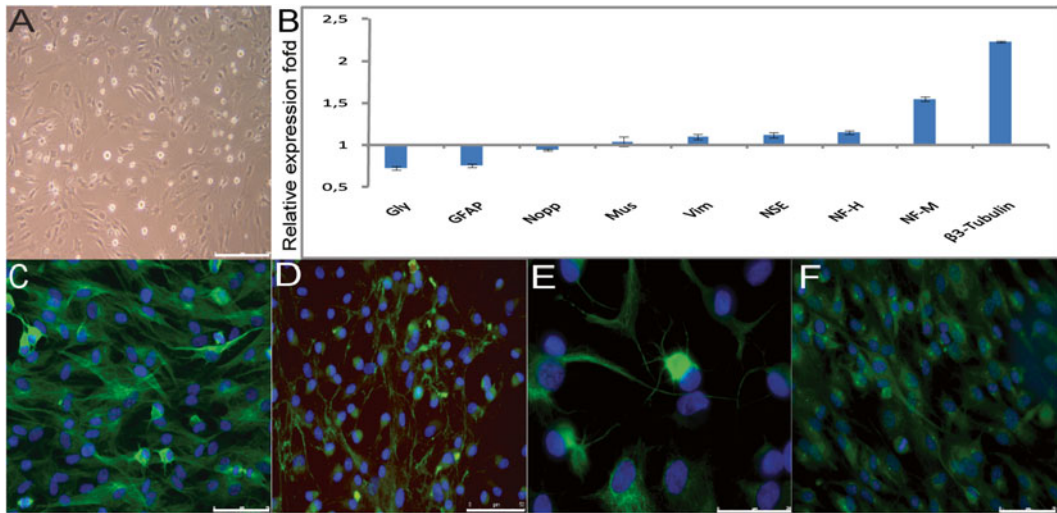


Fig. 5 DPSC neural differentiation characterization. (a) Evidence of the neural differentiated DPSC morphology change, bar scales 150 μm . (b) Semiquantitative rt-PCR was used to determine the gene expression profile ratio of neural specific markers in neural differentiated over undifferentiated DPSC cells. Values were expressed as mean $\pm$ SD and were normalized using the β -actin. X-axis shows relative expression fold and Y-axis tested markers (adjusted $P < 0.05$). (c) FITC-labeled antibody was used to evaluate β^3 -tubulin expression in neural differentiated DPSC, bar scales 75 μm . (d) FITC-labeled antibody was used to evaluate tyrosine hydroxylase expression in neural differentiated DPSC, bar scales 50 μm . (e) FITC-labeled antibody was used to evaluate neurofilament-medium expression in neural differentiated DPSC, bar scales 25 μm . (f) FITC-labeled antibody was used to evaluate glial fibrillar acid protein expression in neural differentiated DPSC, bar scales 75 μm . Nuclei were counterstained with DAPI. Reproduced from [32]

control for Oct4 expression [23]. Human osteoblast-like cells (hOB) are positive control for osteoblastic differentiation [24]. Human primary thyroid cells (HT) are the negative control for osteogenic differentiation [25]. Human supernumerary teeth buds (STB) can be isolated following same methods used for DPSC, and used as odontoblastic positive control [26]. Human hepatocellular carcinoma cells (HepG2) are the positive control for hepatic differentiation [27]. Small fragments of cardiac tissue are positive control for cardiomyocyte (from discarded hearts with permission). Human neuroblastoma (Be2C) cells are the positive control for neural differentiation [28].

4 Notes

1. Do NOT heat.
2. The F12 Coon's and Ambesi's modified culture medium [29] used was a modification [30] of Ham's F-12M [31]. Compared to Ham's F-12, this custom medium has a twofold increase in the amino acid concentration and the addition of sodium pyruvate as well as ascorbic acid.

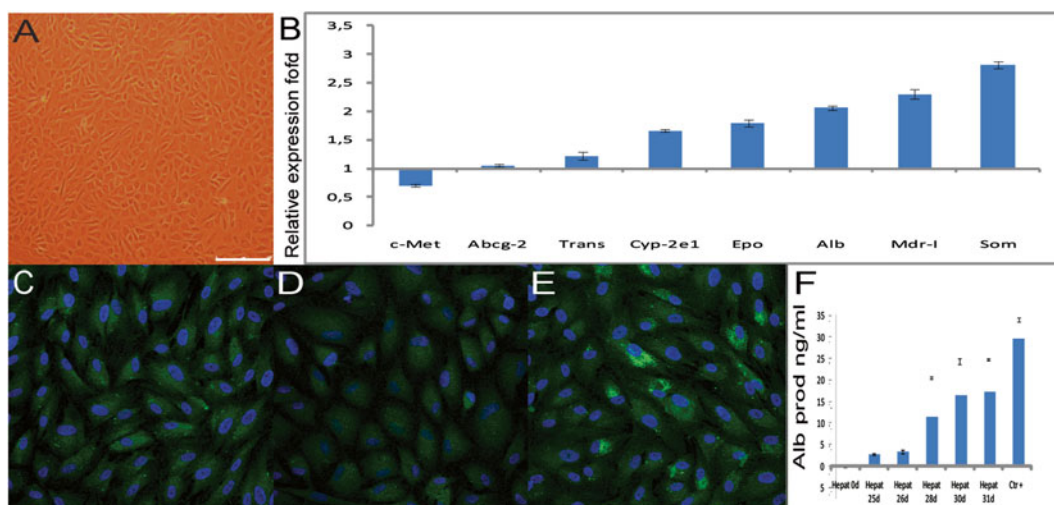


Fig. 6 DPSC hepatocyte differentiation characterization. (a) Evidence of the hepato-differentiated DPSC morphology change, bar scales 150 μ m. (b) Semiquantitative rt-PCR was used to determine the gene expression profile ratio of hepatic specific markers in hepato-differentiated over undifferentiated DPSCs. Values were expressed as mean $\pm$ SD and were normalized using the β -actin. X-axis shows relative expression fold and Y-axis tested markers (adjusted $P < 0.05$). (c) FITC-labeled antibody was used to evaluate cytokeratin 8 expression in hepato-differentiated DPSC, bar scales 75 μ m. (d) FITC-labeled antibody was used to evaluate cytokeratin 18 expression in hepato-differentiated DPSC, bar scales 75 μ m. (e) FITC-labeled antibody was used to evaluate cytokeratin 19 expression in hepato-differentiated DPSC, bar scales 75 μ m. Nuclei were counterstained with DAPI. (f) ELISA assay showed a time-dependent increased albumin secretion in the medium from 11 ng/mL on day 25 to 25 ng/mL on day 31 after hepatic differentiation. Values were expressed as mean $\pm$ SD of three independent experiments. X-axis secreted albumin concentration (ng/mL) and Y-axis differentiation days (adjusted $P < 0.05$). Reproduced from [32]

3. Medium performance is maximal within 2 weeks; after this period medium performance decreases due to instability of the growth factors.
4. When agar is cooled add 5 mL of sterile 1 $\times$ PBS to completely cover agar in the culture dish; this prevents the agar from becoming sticky.
5. If the tooth is left in an empty container dental pulp isolation must be done within 4–6 h. Teeth with small and closed pulp cavities, such as incisors and canines, will be better conserved in empty containers. Maintaining the tooth in milk extends the storage time to 1 day and prevents open pulps from drying out, but isolating tissue sooner is always preferable.
6. Alternatively, other researchers submit dental pulps to enzymatic digestion; however, we find that this technique results in some loss of cells during centrifuging and washing processes.

7. We have determined that 1.5 mL of medium is optimal in order to completely submerge pulp fragments and at the same time enable pulp contact to culture dish in order to facilitate adhesion and colonization of the cells onto the plate.
8. Warming the medium to 37 °C prior to use is necessary in order to avoid additional stress on dental pulps cells.
9. On the contrary splitting will reduce, hypothetically by 50 %, the number of DPSC progenitor cells and will delay primary culture start.
10. It is very important not to move the culture dishes for 3 days in order to allow the dental pulp tissue to attach to the tissue culture plate and the cells to outgrow and proliferate.
11. Do not allow cells to dry during medium change.
12. Viable DPSC primary culture will adhere to the bottom of the plate. If DPSCs are largely in suspension, appearing as enlarged round cells that are not refracting light around their membrane, the primary culture has died.
13. Because of the low serum concentration in these cultures it is not necessary to perform a PBS wash step to remove serum prior to adding the CTC cell detachment solution.
14. Alternatively lightly tap the bottom of the conical tube to resuspend cells.
15. Avoid bubble formation.
16. Rotate culture dish 3–5 times to homogeneously distribute cells onto culture surface.
17. Culture should be maintained at a semi-confluent condition in order to prevent the differentiation of the cells.
18. Prevent bubbles and resuspend cells as single-cell suspension to obtain a true count, typically, optimal cell concentration between 250,000 cells/mL and 2.5 million cells/mL. Above 2.5 million, dilute sample to obtain a final concentration closer to the optimum.
19. The aim of cryopreservation is to enable stocks of cells to be stored to prevent the need to have all cell lines in culture at all times, reduce risk of microbial contamination, reduce risk of genetic drift and morphological changes, and permit to work with cells at a consistent passage number.
20. Rotate culture dish 3–5 times to homogeneously distribute cells onto culture surface; make sure to avoid the formation of bubbles under cover slip.
21. Alternatively during the twice-a-week medium change, leave in the plate 1/7 volume of conditioned medium to improve differentiation results.

22. Cells compact and form a three-dimensional (3D) ordered structure within 1 week. However, media change during the first 7 days of culture will disrupt the cell compaction phase and damage the aggregate.

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Chapter 9

Dental Pulp Stem Cells Isolation and Osteogenic Differentiation: A Good Promise for Tissue Engineering

Adriana Di Benedetto, Claudia Carbone, and Giorgio Mori

Abstract

Adult stem cells therapy can be an efficacious treatment for many diseases and disabilities. New sources of stem cells in adult organisms are continuously emerging. Dental tissues that are easily accessible by a tooth extraction have been identified as a source of postnatal mesenchymal stem cells capable of self-renewal and multipotency. Here, we describe accurately the technical procedure to isolate mesenchymal stem cells from dental pulp (DPSCs), characterize their immunophenotype, and assay their osteogenic capacity.

Key words Mesenchymal stem cells, Dental tissues, Dental pulp stem cells, Osteogenic differentiation, Regenerative medicine

1 Introduction

Stem cell research goes far beyond the scientific and therapeutic potential of regenerative medicine [1–5]. The adult stem cells found in permanent and in deciduous teeth provide the prospect for bone, dentin, and other tissues regeneration; in particular, opportunely cultured on viable scaffolds, dental stem cells could be used for the replacement of missing bone and cartilage [6]. Tooth banking is based on the firm belief that bioengineered tissue could be the most promising resource for treating challenging diseases and injuries that would occur throughout life. Individuals have many opportunities at different stages of their life for banking their valuable cells. In particular, dentists are at the forefront of engaging their patients in potentially life-saving therapies derived from their own stem cells located in dental tissues [7]. Dental stem cells are a source of adult stem cells that are easily accessible by tooth extraction or when a primary tooth is replaced [8]. The third molar erupts relatively late in human life, is often useless and sometimes a detrimental organ, thus representing the ideal source to collect dental stem cells.

Many different types of human dental stem cells have been well described in the literature: (1) Dental pulp stem cells (DPSCs) [6, 9]; (2) Stem cells isolated from human exfoliated deciduous teeth (SHEDs) [10]; (3) Periodontal ligament stem cells (PDLSCs) [11]; (4) Stem cells isolated from the apical papilla (SCAPs) [12]; (5) Stem cells isolated from dental follicle (DFSCs) [13]. Within the body, mesenchymal stem cells (MSCs) have been localized to perivascular niches [14, 15] and recent studies have shown that dental stem cells are also localized to perivascular niches within the tooth structure [16–18]. Dental stem cells arise from dental mesenchyme which has early interaction with the neural crest during normal tooth development. Therefore, dental stem cells may display characteristics of both mesoderm and ectoderm due to their ectomesenchymal origins [19]. In this chapter we describe the methods to isolate DPSCs from wisdom tooth, and differentiate them toward the osteoblastic phenotype. DPSCs represent an exciting cell source for applications in the regeneration of tissues in dentistry and are easily obtainable from the dental pulp of teeth that are often discarded as waste [20]. Within the next few years, stem cells will be used to restore the form and function of the oral cavity using autologous cells, thereby overcoming histocompatibility mismatch. While we can see the promise of human stem cell therapies for the future, dentists know that it is important to act now to harvest and store DPSCs because the opportunity to bank patient's dental stem cells could have a greatest future impact. Thus, stem cells seized in young and healthy people, could be eventually used for future regenerative therapies in elderly or during disease occurrence. The potential of dental stem cells as an alternative choice to embryonic stem cells seems realistic for future stem cell therapies and regenerative medicine [21–25]. DPSCs are a heterogeneous population of cells that were first isolated by Gronthos et al. [6] and exhibited some characteristics of bone marrow mesenchymal stem cells (BMMSCs), including the production of fibroblast-like cells that were clonogenic and had a high proliferation rate. Interestingly, DPSCs showed higher proliferation rate than BMMSCs. DPSCs also had a similar protein expression pattern to BMMSCs in vitro including vascular adhesion molecule 1, alkaline phosphatase, collagen I, collagen III, osteonectin, osteopontin, osteocalcin, bone sialoprotein, α -smooth muscle actin, fibroblast growth factor 2, and the cell surface marker CD 146 [6, 26]. Immunohistochemistry staining further showed that like BMMSCs, primary cultures of DPSCs did not stain for the cell surface markers CD14, CD34, and CD45 or other markers including Myo D, neurofilament, collagen II, and peroxisome-proliferator activated receptor γ -2 [6]. FACS (fluorescence activated cell sorting) has been recently used to sort DPSCs based on cell surface markers founding that in addition to the above identified markers, DPSCs also expressed the following: CD9, CD10, CD13, CD29, CD44,

CD49d, CD59, CD73, CD90, CD105, CD106, CD166, and STRO-1 [27–29]. Further, DPSCs did not express CD14, CD31, or CD45 [28]. When cultured under osteogenic conditions DPSCs were capable of forming calcified deposits sparsely throughout the culture and their spectra, analyzed by FT-IR microscopy, changed phosphate stretching vibrations [30]. In vivo transplantation of DPSCs into immunocompromised mice resulted in the production of a dentin-pulp-like complex with a collagen matrix containing blood vessels and lined with odontoblasts, suggesting that DPSCs are multipotent. Further studies also found DPSCs to be multipotent, capable to differentiate into myoblasts, osteoblasts, odontoblast-like cells, chondrocytes, adipocytes, and neural cells [31, 32].

2 Materials

For isolation, cultivation, and characterization of human DPSCs were used the following listed equipment, solutions, and materials. All the materials used for cell culture must be sterile, in all the steps. Use deionized water to make diluted solutions unless differently specified.

2.1 Equipment

1. Centrifuge.
2. Laminar flow hood.
3. CO₂ incubator; 37 °C and 5 % CO₂.
4. Light microscope.
5. Spectrophotometer.
6. Agitator.
7. Flow cytometer.
8. Hemocytometer.

2.2 Reagents

1. Mouthwash.
2. Cotton balls.
3. 0.2–0.3 % chlorhexidine.
4. Betadine.
5. Accutase.
6. 70 % ethanol.
7. Dental fissure burs.
8. Sterile scalpels.
9. Sterile pincers.
10. Serological pipettes.
11. Micropipettes.

12. Sterile pipette tips.
13. Sterile 15 and 50 mL tubes.
14. 70 μ m cell strainers.
15. T-25 or T-75 sterile flasks.
16. FACS tubes.
17. 12 multi-well culture plates and 96 multi-well culture plates.
18. Fluorochrome-conjugated antibodies.

2.3 Media

1. α -MEM supplemented with 100 U/mL penicillin-G, 100 μ g/mL streptomycin.
2. Pulp digesting medium (PDM) composed of α MEM, 100 U/mL penicillin-G, 100 μ g/mL streptomycin, 3 mg/mL Type I collagenase plus, 4 mg/mL Dispase (*see Note 1*).
3. Mesenchymal Stem Cell Culture medium (provided by different companies) supplemented with 5 % heat inactivated fetal bovine serum (FBS), 100 U/mL penicillin-G, 100 μ g/mL streptomycin.
4. Sterile phosphate buffered saline (PBS): 80 g NaCl, 2.0 g KCl, 14.4 g Na_2HPO_4 , 2.4 g KH_2PO_4 in 1 L of deionized water. The pH was adjusted to 7.2, the solution was sterilized by autoclaving, let cool and supplemented with 100 U/mL penicillin-G, 100 μ g/mL streptomycin (*see Note 2*).
5. FACS Buffer (PBS pH 7.2 and 0.5 % bovine serum albumin (BSA)) (*see Note 2*).
6. Osteoblast differentiation medium (α -MEM, 100 U/mL penicillin-G, 100 μ g/mL streptomycin, supplemented with 2 % FBS, 50 μ g/mL ascorbic acid, and 10^{-8} M dexamethasone) (*see Note 3*).
7. Mineralization medium (Osteoblast differentiation medium supplemented with 10 mM β -glycerophosphate) (*see Note 3*).
8. Leukocyte Alkaline Phosphatase Kit (all reagents including fixative solution are included in the kit).
9. Von Kossa Staining: 10 % Formalin in PBS (*see Note 4*), 5 % AgNO_3 , 5 % sodium thiosulfate (*see Note 5*) in deionized water.
10. Alizarin Red S staining (ARS): PBS (*see Note 2*), 10 % Formalin (*see Note 4*), ARS powder 1 % in deionized water (*see Note 6*), 10 % acetic acid, 10 % ammonium hydroxide (*see Note 7*).

3 Methods

3.1 Dental Pulp Extraction

1. Human dental pulps were extracted from wisdom teeth of young adult healthy volunteers undergoing orthodontic treatments. Before the tooth extraction the whole oral cavity was

rinsed with mouthwash to lower the bacterial load. Then the dental crown was locally disinfected with a cotton ball imbibed of 0.2–0.3 % chlorhexidine (*see Note 8*).

2. After the extraction, the entire tooth surface was well cleaned and disinfected with betadine and 70 % ethanol (*see Note 9*).
3. The tooth was then cut at the cementum–enamel junction by using sterilized dental fissure burs in order to expose the pulp chamber; the cooling was performed with sterile saline solution.
4. The pulp tissue was gently separated with sterile scalpels and pincers from the crown and the root canals and stored in sterile tubes at 4 °C for maximum 5 h in α MEM, 100 U/mL penicillin-G, 100 μ g/mL streptomycin until cells isolation procedure.
5. Only healthy dental elements, without carious disease or hyperemic pulp tissue, were selected. The study was approved by the Institutional Review Board of the Department of Clinical and Experimental Medicine, Dental Clinic, University of Foggia. Patients gave written informed consent.

3.2 Dental Pulp Stem Cell Isolation

1. Each individual dental pulp obtained from different donors was placed in sterile culture dish and diced into as small as possible pieces under sterile conditions in a laminar flow hood using a sterile scalpel.
2. Pulp pieces were transferred with sterile pincers and immersed in 5 mL of PDM in a 50 mL sterile tube for enzymatic digestion. The tube was hold on the agitator posted in the incubator for 1 h (*see Note 10*). The agitator was set at 250 rpm.
3. After 1 h 5 mL of α MEM supplemented with 5 % FBS was added to digested pulp suspension to neutralize the action of collagenase and dispase. Suspension was collected with a sterile pipette and filtered through a 70 μ m cell strainer. The 70 μ m cell strainer was hold on the top of a new 50 mL collection tube and the suspension containing the digested pulp was filtered through the strainer in order to obtain a single cell suspension (*see Note 11*).
4. The single cell suspension was collected at the bottom of the 50 mL collection tube and centrifuged for 5 min at $1500 \times g$. The pellet was resuspended in Mesenchymal Stem Cell Culture medium supplemented with 5 % heat inactivated FBS, 100 U/mL penicillin-G, 100 μ g/mL streptomycin and plated at 5×10^3 cells/cm² (*see Note 12*).
5. Approximately 24 h after plating, the cells were able to adhere to the plastic and after 48–72 h acquired the typical colony-forming unit appearance. The cells within each colony appeared characterized by a typical fibroblast-like morphology (Fig. 1)

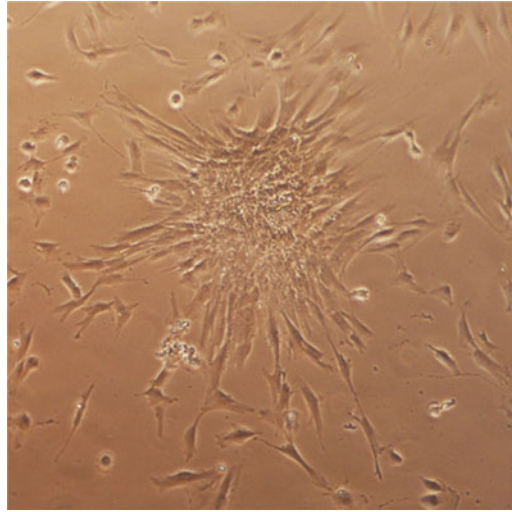


Fig. 1 In vitro appearance of cells isolated from dental pulp and cultured as described (40× magnification). 24 h after plating, the cells were able to adhere to the plastic and after 48–72 h acquired the typical colony-forming unit appearance. Cell morphology is typical fibroblast-like

analogous to the human bone marrow CFU-F [6, 33]. The cell culture medium was replaced every 48 h. When the cells reached 90 % of confluence (*see Note 13*), we split and expanded not over the passage V (*see Note 14*).

6. On each splitting, the cell culture medium was discarded and the cells were washed once with PBS to remove serum residues. PBS was discarded from the flask, Accutase was added and the flask placed in incubator at 37 °C and 5 % CO₂ for 2–3 min (*see Note 15*).
7. Accutase was neutralized with same volume of cell culture medium and the cells were collected with a serological pipette in a sterile tube. Cell suspension was centrifuged for 5 min at 1,400 rpm and resuspended accordingly to the cell density indicated above or used for downstream assays.

3.3 Characterization of DPSCs

Fluorescence Activated Cell Sorting (FACS), also called flow cytometry, has been widely used for the immunophenotypic characterization of mesenchymal stem cell subpopulations from different sources. In several studies, cells isolated from dental pulp were sorted basing on cell surface or intracellular markers and showed to meet the minimal criteria to be defined as mesenchymal stem cells [2]. DPSCs expressed the main surface markers identified in mesenchymal stem cell subpopulations, as CD 73, CD 90, CD 105, CD 13, CD 29, CD 44, HLA-I, while failed to react with the hematopoietic markers CD14, CD45, CD34 [6, 27–29, 34].

For this characterization, the cells were detached as described in Subheading 3.2 and counted with a hemocytometer.

1. To test each marker, we used approximately 1×10^6 nucleated cells resuspended in 90 μL of FACS buffer in appropriate FACS tubes.
2. Each cell aliquot was incubated with 10 μL of each fluorochrome-conjugated antibody (*see Note 16*), mixed well by vortexing and incubated for 15 min in the dark at $2-8^\circ\text{C}$ in the refrigerator (*see Note 17*).
3. After incubation the cells were washed with 4 mL FACS buffer and centrifuged at $300 \times g$ for 5 min at 4°C . The Supernatant was completely aspirated and the pellet was resuspended in 500 μL PBS.
4. Data were acquired using a flow cytometer and the results analyzed by a suitable software [35]. DPSCs isolated with this procedure exhibited $\geq 95\%$ expression of CD73, CD90, CD146, CD44, CD105, and HLA-I, while were negative for CD45.

3.4 Osteogenic Capacity

To assess the ability of DPSCs to differentiate in osteoblast and form mineralized nodules in vitro, the cells were cultivated as follow.

1. For induction of osteoblast differentiation, the cells were seeded $3,000/\text{cm}^2$ in 12 multi-well culture plate in presence of osteoblast differentiation media (*see Note 18*).
2. The media was replaced every 3–4 days (*see Note 19*).
3. The culture was stopped on different time points (0, 7, 14, and 21 days) (*see Note 20*) and assayed for the expression of Alkaline Phosphatase (ALP) with Leukocyte Alkaline Phosphatase Kit, based on naphthol AS-BI and fast red violet LB.

3.5 Alkaline Phosphatase (ALP)

1. Cell culture medium was discarded from all wells and the cells were fixed by adding 0.5 mL/well of Fixative Solution provided by the kit for 30 s at room temperature.
2. The wells were rinsed thoroughly in deionized water without allow the cells to dry. 0.5 mL of ALP solution was added into each well and incubated for 15 min at room temperature in the dark.
3. At the end of incubation the ALP solution was discarded, the wells were rinsed in deionized water and air-dried before microscopic analysis.
4. ALP positive cells are stained in purple as represented in Fig. 2. Only the cell populations positive for the expression of Alkaline Phosphatase will be able to form mineralized nodules on appropriate culture conditions. Thus, cell populations assayed

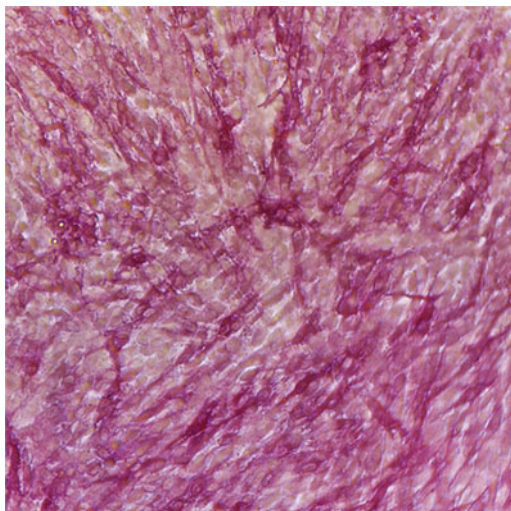


Fig. 2 DPSCs cultured in presence of osteoblast differentiation medium for 1 week, fixed and stained for ALP (40× magnification). Purple cells are positive to ALP expression

for Alkaline phosphatase were also tested for the ability to produce in vitro mineralized matrix nodules.

5. The cells were seeded 3,000/cm² in 12-multi-well culture plate in the presence of mineralization medium (*see Note 18*) and rinsed every 3–4 days (*see Note 19*). The culture was stopped at 14–21 days and assayed for the presence of mineralized matrix with Von Kossa or Alizarin Red staining (*see Note 21*).

3.6 Von Kossa staining

1. The cell medium was discarded and the cells were fixed for 10 min by adding 0.5 mL/well of 10 % Formalin.
2. The cells were rinsed 3× with deionized water, incubated with 5 % AgNO₃ for 30 min in the dark, washed with 5 % sodium thiosulfate, and rinsed again 3× with deionized water.
3. The multi-wells were then exposed to white light for 1 h and inspected by phase microscopy. A typical result is reported in Fig. 3 (*see Note 22*). Dark areas represent calcium salts deposits and can be quantified with suitable software (for example NIH Image J).

3.7 Alizarin Red S staining (ARS)

ARS is an alternative method to detect calcium-rich deposits in cell cultures. In this technique, after microscopic inspection and data acquisition, the dye can be extracted from the stained monolayer and assayed by colorimetric detection at 405 nm [36].

1. The cell culture medium was discarded before staining procedure, the cells were rinsed gently with same volume or more of PBS (*see Note 23*) and fixed for 10 min by adding 0.5 mL/well of 10 % formalin.

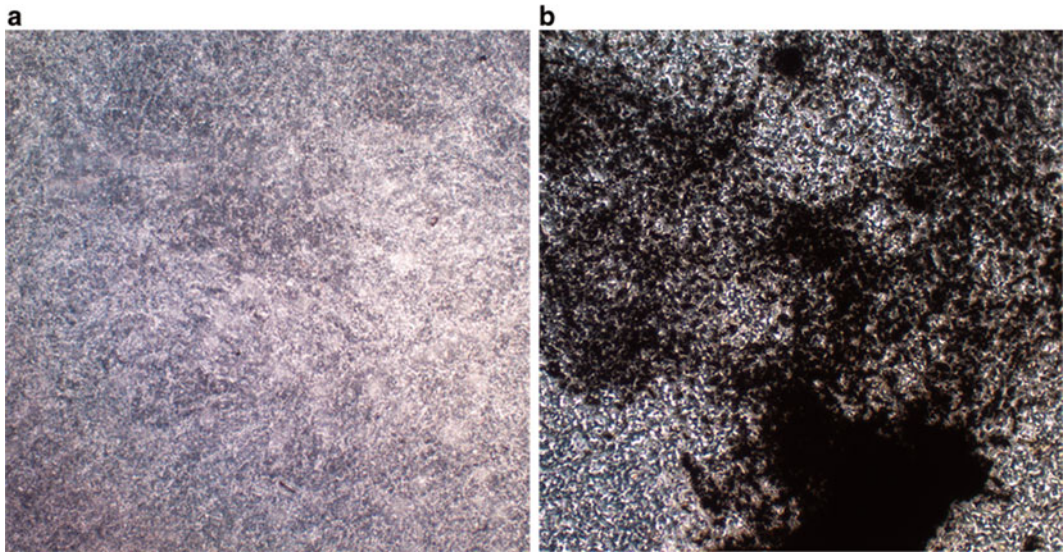


Fig. 3 Von Kossa stained DPSCs cultivated in presence of mineralizing medium for 21 days (20× magnification). At T0 there were no detectable mineral deposits on the confluent monolayer (**a**). After 21 days in mineralizing condition, dense nodules stained in black are visible (**b**)

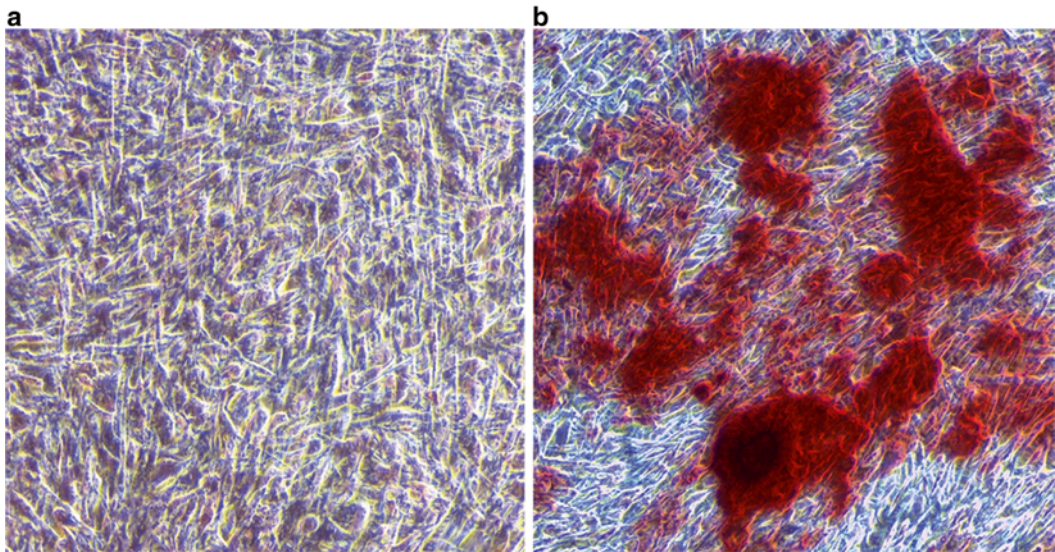


Fig. 4 Large red areas represent the mineral deposits stained with ARS (**b**) (40× magnification). No red detectable staining is observed in control conditions (**a**)

2. Fixative residues were removed by washing 2× with deionized water. 1 % ARS solution was added (0.5 mL/well) and incubated for 10 min at room temperature.
3. ARS solution was discarded, the wells were rinsed twice with deionized water and air-dried. The monolayer appeared densely red stained as reported in Fig. 4.

4. For staining quantification, 800 μL of 10 % acetic acid was added to each well and incubated at room temperature for 30 min with shaking.
5. The cell monolayer was scraped with an appropriate cell scraper and the suspension was transferred by pipetting to a 1.5-mL microcentrifuge tube.
6. After vortexing for 30 s, the solution was incubated 10 min at 85 °C and then kept on wet ice for 5 min (*see* **Note 24**).
7. The suspension was centrifuged at $20,000\times g$ for 15 min, 500 μL of the supernatant were transferred to a new 1.5-mL microcentrifuge tube and 200 μL of 10 % ammonium hydroxide were added to neutralize the acid.
8. 150 μL of the solution were transferred in 96 multi-well plates and read in triplicate at 405 nm. Results were evaluated for statistical analysis.

4 Notes

1. Prepare storage aliquots of Type I collagenase plus (30 mg/mL) and Dispase (10 mg/mL) by diluting the powder in PBS and store at $-20\text{ }^{\circ}\text{C}$. Avoid repeated freezing. Prepare pulp digesting media (PDM) freshly each time, don not store the solution.
2. Store the buffer at $2\text{--}8\text{ }^{\circ}\text{C}$
3. Prepare storage 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 $-20\text{ }^{\circ}\text{C}$. Prepare osteoblast differentiation medium by diluting fresh aliquots of ascorbic acid and dexamethasone in α -MEM, 100 U/mL penicillin-G, 100 $\mu\text{g/mL}$ streptomycin, supplemented with 2 % FBS. Osteoblast differentiation medium must be prepared freshly on each culture feeding. Prepare freshly mineralization medium on each culture feeding by adding fresh aliquots of β -glycerophosphate to osteoblast differentiation medium.
4. Make freshly 10 % formalin by diluting 37 % formaldehyde in PBS.
5. Make freshly 5 % AgNO_3 and 5 % sodium thiosulfate in deionized water.
6. Dissolve 1 % of ARS powder in deionized water by stirring at room temperature, filter the solution through a paper filter held in a funnel, allowing gravity to draw the liquid through the paper and collect the drawn liquid in a glass becker. This procedure helps to remove the solid undissolved particles from the liquid.

Since the filter paper will absorb a significant volume of liquid, be sure to make solution in excess. The solution can be stored at room temperature until precipitate formation.

7. Prepare dilutions in deionized water and store at room temperature.
8. Make repeated washing (at least three) of the oral cavity with commercial mouthwash to reduce the bacterial load of all mouth. Disinfect the dental crown with a cotton ball imbibed of 0.2–0.3 % chlorhexidine hold with sterile pincers.
9. Be aware to not break the tooth and not expose the pulp chamber during the tooth extraction. If the tooth breaks up in the mouth this will facilitate bacterial contamination of pulp tissue and consequently of the cell culture.
10. Alternatively the tube containing the pulp with PDM can be frequently stirred manually, if an agitator is not available.
11. Wash the top of the strainer with extra cell culture medium (α -MEM) or PBS in order to increase the yield of collected cells.
12. Prewarm at 37 °C the medium before the use. Resuspend the cell pellet in 25–75 cm² flasks accordingly to cell density and number.
13. Establish the cell density approximately by microscopic observation. Cell culture confluence of 90 % is reached when is still possible to observe few zones of the flask not covered by cells.
14. Add “one passage” each time the cells are split. DPSCs can lose their stemness properties over passage V [37]. The expansion process induces senescence of MSCs and loss of stemness as shown by a decline in proliferative and differentiation capacity [38, 39].
15. Do not overcome 5 min of incubation with Accutase. Overtime incubation can result in cell lysis and loss of viability. After incubation pipette vigorously many times to favor mechanical cell detachment. Check that cells are detached by microscopic observation. Use Accutase in place of Trypsin. Accutase treatment does not significantly affect the viability and proliferation rate of stem cell dissociation into single cells [40].
16. The suggested volume is for up to 1×10^6 nucleated cells. When working with fewer than 1×10^6 cells, use the same volumes as indicated. If working with higher cell numbers, scale up all reagent volumes and total volumes, accordingly (e.g. for 2×10^6 nucleated cells, use twice the volume of all indicated reagent volumes and total volumes).
17. The recommended incubation temperature is 2–8 °C. Higher temperatures and/or longer incubation times may lead to nonspecific cell labeling. In contrast, working on ice may require increased incubation times.

18. To evaluate the osteoblastic commitment of DPSCs, long-term culture conditions are required therefore a low cell density (minimum 3,000 cells/cm²) and a minimum percentage of FBS in the culture medium are strictly recommended. Indeed osteoblast-like cells grow in multilayer and produce extracellular matrix, thus causing cell layer rolling up and detachment.
19. Pay attention during the cell feeding. Do not aspirate the cell culture medium with vacuum, this will perturb the cell layer and will cause roll up. Micropipette and sterile tips (1,000 µL) are strictly recommended. Aspirate the medium gently, try not to touch the cell layer with the tip, leave a thin film of liquid in each well before adding the fresh medium. These devices will help to keep the culture for long time avoiding cell rolling up.
20. Seed a 12-multi-well culture plate with cells in the presence of Mesenchymal Stem Cell Culture Medium supplemented with 5 % of FBS for the time 0 and stop it for ALP staining 24–48 h later. Seed a 12-multi-well culture plate for each time point (7, 14, and 21 days) and stop it at different time points for staining. Seed at least three wells for each time point or condition for statistical analysis.
21. Seed a 12-multi-well culture plate with cells in presence of Mesenchymal Stem Cell Culture Medium supplemented of 5 % of FBS for the time 0 and stop it for appropriate staining 24–48 h later. Seed a 12-multi-well culture plate for each time point (14 and 21 days; you will not be able to see enough mineralized matrix before) and stop it at different time points for staining. Seed at least three wells for each time point or condition for statistical analysis.
22. Store the plate in the dark. Prolonged light exposure will cause staining modification.
23. It is important to wash the cells with same PBS volume as culture medium in the wells or more. This will remove culture medium residues also from the lateral side of the well, avoiding aspecific staining. This is particularly important in the colorimetric detection.
24. Take care in this phase to avoid opening of the tubes.

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Chapter 10

Efficient Hepatic Differentiation of Human Induced Pluripotent Stem Cells in a Three-Dimensional Microscale Culture

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Abstract

Human induced pluripotent stem cells (iPSCs) represent a novel source of hepatocytes for drug development, disease modeling studies, and regenerative therapy for the treatment of liver diseases. A number of protocols for generating functional hepatocytes have been reported worldwide; however, reproducible and efficient differentiation remained challenging under conventional two-dimensional (2D) culture. In this study, we describe an efficient differentiation protocol for generating functional hepatocyte-like cells from human iPSC-derived homogenous hepatic endoderm cells combined with three-dimensional (3D) microscale culture system. First, hepatic endoderm cells (iPSC-HEs) were directly differentiated using two-step approaches, and then cultured in the 3D micropattern plate. Human iPSC-HEs quickly reaggregated and formed hundreds of round-shaped spheroids at day 4 of cell plating. The size distribution of iPSC-HEs derived spheroids was relatively uniform around 100–200 μm in diameter. After 14 days, iPSC-HEs efficiently differentiated into hepatocyte-like cells in terms of hepatic marker gene expression compared with conventional 2D approach. We conclude that our scalable and three-dimensional culture system would be one promising approach to generate a huge number of hepatocyte-like cells from human iPSCs aiming at future industrial and clinical applications.

Key words Human induced pluripotent stem cells, Hepatocyte-like cells, Three-dimensional culture, Spheroid

1 Introduction

A great deal of cost in pharmaceutical industry reach billions of dollars per year due to significant drug failures, followed by nearly one-third of drugs withdrawn from the market [1, 2]. This is partially explained by the lack of predictive preclinical models for drug safety and efficiency testing. Current systems for predicting humanized drug metabolism profiles in vitro mainly depend on the use of isolated primary human hepatocytes [3, 4]. However, there have

been major drawbacks for modeling drug metabolism assays [5–7]. Firstly, the shortage of healthy donor organ as well as incredibly high cost restricts its real-world application in pharmaceutical industries; secondly, shipped human hepatocytes display batch-to-batch variation in functionality; thirdly, human hepatocytes isolated from liver with low viability showed loss of CYP3A4 and CYP2B6 expression which could contribute to the formation of metabolites; and fourthly, they are not stable in *in vitro* culture for more than 2–3 weeks and their ability to secrete albumin decreased [8, 9, 5, 10, 11].

Recently, there has been a remarkable progress in the field of the reprogramming skills to derive pluripotent stem cells, or induced pluripotent stem cells (iPSCs), from somatic cells by defined factors [12, 13]. Not only can iPSCs be propagated almost indefinitely, but they also have the capacity to differentiate into every cell and tissue type, which comprises the fully developed organism [14, 15]. The use of human iPSC-derived hepatocyte-like cells (iPSC-Heps) attract much interest of pharmaceutical industry due to the advantage of reproducible and stable quality as well as low cost compared with that of conventional adult hepatocytes [16, 17]. A number of studies have been made towards optimizing protocols for the generation of hepatocyte-like cells from induced pluripotent stem cells over the past few decades [18–20]. Almost all approaches have sequentially added the key inductive factors into the iPSC-derivatives under two-dimensional culture to mimic the molecular process of hepatogenesis. Unfortunately, differentiated cells significantly lack the functional characteristics of mature hepatocytes including drug metabolism capacity, and as a consequence, those produced populations in most case represent immature hepatocytes. Furthermore, the much variability and inefficiency of this process is considered a practical barrier to apply them into a drug development field, since a drug assay requires plenty of functional cells in a stable quality with excellent drug metabolic enzyme activity [21].

Here, we will present a three-dimensional (3D) culture protocol to stimulate efficient hepatic maturation of human iPSCs by allowing cells interact three-dimensionally, as is the importance of dynamic intercellular interactions becoming clear in recapitulating proper organogenetic process [22, 23, 21, 24]. After hepatic specification of human iPSCs (iPSC-HEs), cells were detached and reagggregated in a microscale culture plate to promote the maturation into hepatocyte-like cells [25]. Uniformly generated microspheroids significantly upregulated hepatic marker gene compared with conventional protocols. Also we will describe a high-throughput and quantitative morphological analysis on a large number of iPSC-HEs derived spheroids based on the fluorescence imaging. These generated spheroids containing hepatocyte-like cells might be a powerful tool for predicting humanized profiles of drug metabolism.

2 Materials

2.1 Reagents

2.1.1 Mouse Embryonic Fibroblasts (MEF)

Preparation

1. MEF: mouse embryonic fibroblast cells from E13.5.
2. MEF culture media: D-MEM high glucose was supplied with 10 % FBS, 1 % penicillin–streptomycin, store at 4 °C.
3. 0.1 % gelatin/distilled water, cell culture grade, sterile.

2.1.2 Human iPSCs Maintenance

1. We primarily used the TkDA3 human iPSC line. For imaging experiments, GFP knock-in reporters for the expression of adeno-associated virus integration site 1 (AAVS1::GFP) were used.
2. Dissociation solution: A total volume of 200 ml of dissociation solution contains 20 ml of 2.5 % trypsin, 40 ml of KNOCKOUT™ Serum Replacement (KSR; Gibco), 2 ml of 100 mM CaCl₂, and 138 ml of PBS (*see Note 1*).
3. Human iPSCs media: DMEM/F12 (1:1) supplemented with 25 % KSR, 2 mM GlutaMAX™ Supplement, 1 × MEM nonessential amino acid, and 0.1 mM 2-mercaptoethanol, store at 4 °C.
4. 5 µg/ml human basic fibroblast growth factor (FGF2), stock in –30 °C.
5. Sterile PBS, room temperature.

2.1.3 Human iPSCs Differentiation into iPSC-HE Cells

1. Accutase, stock in –30 °C.
2. Pure DMEM/F12 (1:1), store at 4 °C.
3. RPMI-B27 ΔInsulin media: RPMI 1640 was supplied with 1 % penicillin–streptomycin, 1 % of B-27™ supplement minus insulin (B27 ΔInsulin), store at 4 °C.
4. RPMI-B27 media: RPMI 1640 was supplied with 1 % penicillin–streptomycin, 1 % of B-27™ supplement containing insulin (B27), store at 4 °C.
5. 100 µg/ml recombinant Human/Mouse/Rat Activin A, stock in –30 °C.
6. Matrigel working solution: dilute 1 ml of Matrigel™ Matrix Growth Factor Reduced (GFR) to 29 ml of precooled pure DMEM/F12, store at 4 °C.
7. 10 µM Rock Inhibitor Y-27632, stock in –30 °C.
8. 5 µg/ml human basic fibroblast growth factor (FGF2), stock in –30 °C.
9. 10 µg/ml bone morphogenetic factor 4 (BMP4), stock in –30 °C.
10. Trypan blue stain.

2.1.4 Aggregation of Human Hepatocyte-Like Spheroids in 3D Culture

1. 0.05 % Trypsin–EDTA (Gibco).
2. HCM media: 500 ml of Hepatocyte Basal Medium (HBM™ Medium) and the following culture supplements: Ascorbic Acid, 0.5 ml; Bovine Serum Albumin–Fatty Acid Free (BSA-FAF), 10 ml; Hydrocortisone, 0.5 ml; Transferrin, 0.5 ml; Insulin, 0.5 ml; Gentamicin/Amphotericin-B (GA), 0.5 ml; 5 ng/ml human recombinant HGF; 20 ng/ml recombinant human Oncostatin M (OSM); 100 nM dexamethasone; 5 % FBS (CELLect GOLD, MP Biomedicals), store at 4 °C.
3. DMEM/F12 (1:1) (Gibco) plus 10 % FBS (CELLect GOLD, MP Biomedicals), store at 4 °C.
4. 3D culture plates: EZSPHERE™ (IWAKI, code:4810-900).

2.1.5 Evaluation of Generated Spheroids by IN Cell Analyzer 2000

1. IN Cell Analyzer 2000.
2. IN Cell Developer Toolbox 1.9.

2.2 Equipment

1. Biosafety cabinet.
2. 37 °C, 5 % CO₂ incubator.
3. Centrifuge for 15 and 50 ml tubes.
4. 60 mm culture dish.
5. 100 mm culture dish.
6. Liquid Nitrogen storage tank.
7. 4 °C refrigerator.
8. –20 °C freezer.
9. –80 °C freezer.
10. Pipet-Aid.
11. 37 °C water bath.
12. Phase contrast microscope.
13. Fluorescence microscope.

3 Methods

All procedures are carried out at room temperature except specifically notified otherwise.

3.1 MEF Plating

1. Coat 100 mm polystyrene cell culture dish with 4 ml of 0.1 % gelatin solution (*see Note 2*).
2. Leave at room temperature for about 15 min.
3. Warm MEF culture media at 37 °C water bath.
4. Remove MEF (~5 × 10⁶ cells per vial) from liquid N₂ tank and hold in 37 °C water bath until the sides get thawed but the center is still frozen.

5. Quickly pour the cells into 15 ml conical tube with 9 ml pre-warmed MEF culture media, centrifuge at 900 rpm, 5 min.
6. Remove supernatant and then gently resuspend cell pellet with 10 ml of MEF culture media.
7. Aspirate the coating solution and plate $\sim 0.75 \times 10^6$ cells/100 mm culture dish.
8. Incubate at 37 °C, 5 % CO₂ and change medium as soon as the cells are attached.

3.2 Thaw Human iPSCs Vial and Passage Human iPSCs

1. Set up a 15 ml conical tube. Add 9 ml of pre-warmed human iPSCs media (*see Note 3*).
2. Partially thaw the frozen vial of iPSCs at 37 °C, until there is a small piece of ice remaining. Spray the vial with 70 % ethanol to sterilize (*see Note 4*).
3. Taking 1 ml of pre-warmed media by PIPETMAN at a time, slowly add the media dropwise to the vial and transfer the liquid content with cells into former 15 ml tube.
4. Centrifuge at 770 rpm for 3 min.
5. Meanwhile, wash with PBS one 100 mm dish that was plated with MEFs coated with gelatin 1-day prior. Add 9 ml human iPSCs media (*see Note 5*).
6. Aspirate the media from the spun down tube and gently resuspend the pellet with 1 ml of human iPSCs media. Pipet slowly 1 or 2× maximum, trying to avoid disrupting the chunks of cells, and transfer to MEF plated dish.
7. Incubate at 37 °C, 2.5 % CO₂ overnight, and change the media every 24 h.
8. Colonies should emerge anywhere from 3 to 6 days (*see Note 6*).
9. Take the confluent cells from incubator, washed with 4 ml PBS and removed (*see Note 7*).
10. Add 1 ml of dissociation solution; incubate at 37 °C incubator for 5–8 min (*see Note 8*).
11. Aspirate off the dissociation solution; wash slowly with 2 ml of pre-warmed human iPSCs media to neutralized trypsin contents; and remove the wash media.
12. Nudge the corner of colony 1 ml of human iPSCs media once a time by PIPETMAN to completely detach the colony into small pieces until to a volume of 10 ml.
13. Plating 0.5 ml cells contained media each into 100 mm dish of MEFs that was pre-washed with PBS and containing 9 ml of human iPSCs media.
14. Culture in 37 °C, 2.5 % CO₂ incubator with daily media change.

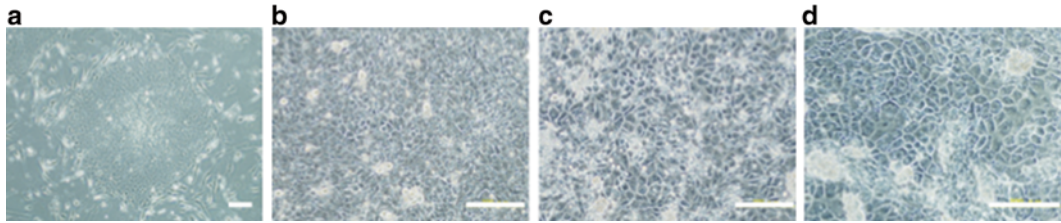


Fig. 1 Hepatic specification of human induced pluripotent stem cells under 2D culture. **(a)** Human iPSCs colony under culture at day 4. **(b)** Human iPSCs plated on Matrigel coated dish at day 1. **(c)** Definitive endoderm cells (iPSC-DE) induced with Activin A treatment for 6 days. **(d)** Hepatic endoderm cells induced with FGF2 and BMP4 treatment for 3 days from the iPSC-DE. Scale bars: 200 μm

3.3 Initiation of Human iPSCs Differentiation

1. Take Matrigel working solution from 4 °C refrigerator and keep it on ice, add 2 ml each into 60 mm dish (*see Note 9*).
2. Incubate at room temperature for 2 h; transfer the Matrigel solution to the former tube for recycle usage, and rinse with pure DMEM/F12 (without any additive factor) until use.
3. Take the human iPSCs from incubator, aspirate the media of human iPSCs, and add 1.5 ml of Accutase (Fig. 1a) (*see Note 10*).
4. Incubate the cells at 37 °C incubator for 5 min until the cells are in a single cell suspension.
5. Harvest the cells into 50 ml conical tube and add pure DMEM/F12 media for wash. Centrifuge the cells for 5 min at 770 rpm in pure DMEM/F12 media to remove any remaining Accutase solution.
6. Resuspend the cells with human iPSCs media contained 5 ng/ml FGF2 and 10 nM Rock Inhibitor Y-27632; plate the cells onto former Matrigel coated dishes with a cell density of $5.0\text{--}8.0 \times 10^4$ cells/cm².
7. Incubate the cells at 37 °C, 5 % CO₂ incubator for growth.

3.4 Differentiation of Human iPSCs into iPSC-HEs

1. Day 1–day 6, take the cells of Subheading 3.3, step 7 (Fig. 1b); aspirate the media, and wash with sterile PBS and remove. Replace with RPMI-B27 Δ Insulin media supplemented with 100 ng/ml of Activin A. Continue to culture for an additional 5 days with media change every 2 days at 37 °C, 5 % CO₂ (Fig. 1c) (*see Note 11*).
2. Day 7–day 10, remove the old media, wash with PBS once, and change medium with RPMI-B27 media supplemented with 10 ng/ml FGF2 and 20 ng/ml BMP4. And culture for another 3 days with media change every 2 days (Fig. 1d) (*see Note 12*).

3.5 Generation of Spheroids from iPSC-HEs in 3D Culture Plates

1. Take the induced iPSC-HEs from Subheading 3.4, step 2, wash with 2 ml PBS twice and remove. Add 1 ml of 0.05 % Trypsin–EDTA, incubate at room temperature for 3 min, and after the cells have detached from the dish, add 2 ml of fresh

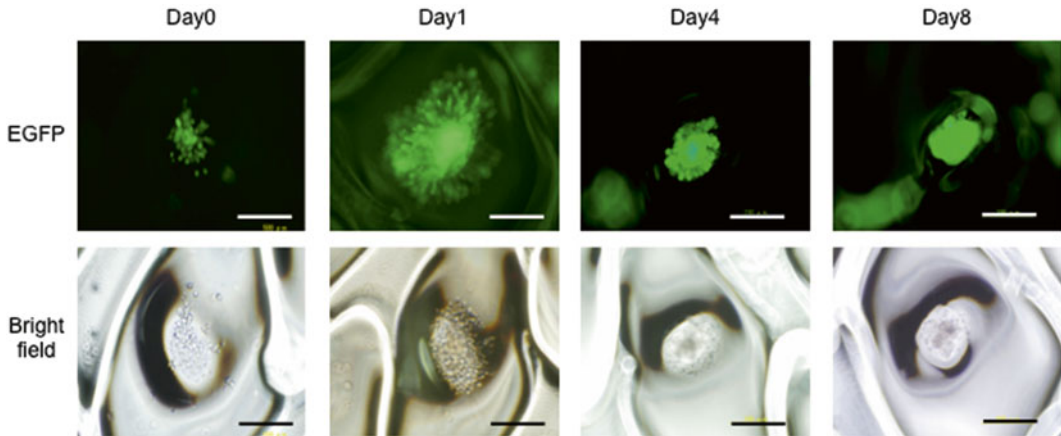


Fig. 2 Generation of human iPSC-HE derived spheroids seeded on 3D micropattern plate. Time course dependent changes in spheroid morphology are shown along with differentiation culture. Scale bars: 200 μ m

DMEM/F12 plus 10 % FBS into the dish. Transfer the cell mixture into a new 15 ml conical tube with 7 ml of DMEM/F12 plus 10 % FBS. Get cell pellet by centrifuging at 800 rpm, for 5 min, at 4 °C.

2. Aspirate the supernatant, resuspend the cell pellet with 10 ml of HCM media, do cell count, and adjust the cell density to 5×10^5 cells/ml (*see Note 13*).
3. Wash the MPC coated EZSPHERE™ plate with PBS, plating 2 ml of the cell suspension into the 3D microsphere culture plate.
4. Incubate the cells at 37 °C, 5 % CO₂.

3.6 Differentiation of Hepatocyte-Like Cells in 3D Microsphere Culture

1. Change medium of cells from Subheading 3.5, step 5 every 2 days by replacing half old medium with half fresh medium (*see Note 14*).
2. Visible hepatocyte-like cells containing spheroids with round edge would be seen 4 days later. Take photos of the generated spheroids every day by fluorescence microscope to trace the sphere size variation (Fig. 2).

3.7 Evaluation of Generated Spheroids by IN Cell Analyzer 2000

1. After 14 days culture, scan the whole well of the assay plates by the IN Cell Analyzer 2000. The instrument is equipped with a 2× dry objective and 488-nm EGFP excitation filter. The plates are exposed for 0.5 ms, and a binning of 3×3 was used for spheroid count assay.
2. Generate composite images from the multiple field of whole well scanning by using the image overlap and stitching algorithm in the IN Cell Developer Toolbox 1.9 software.
3. To monitor the sphere location, EGFP expression images are analyzed using our original algorithm. The algorithm is

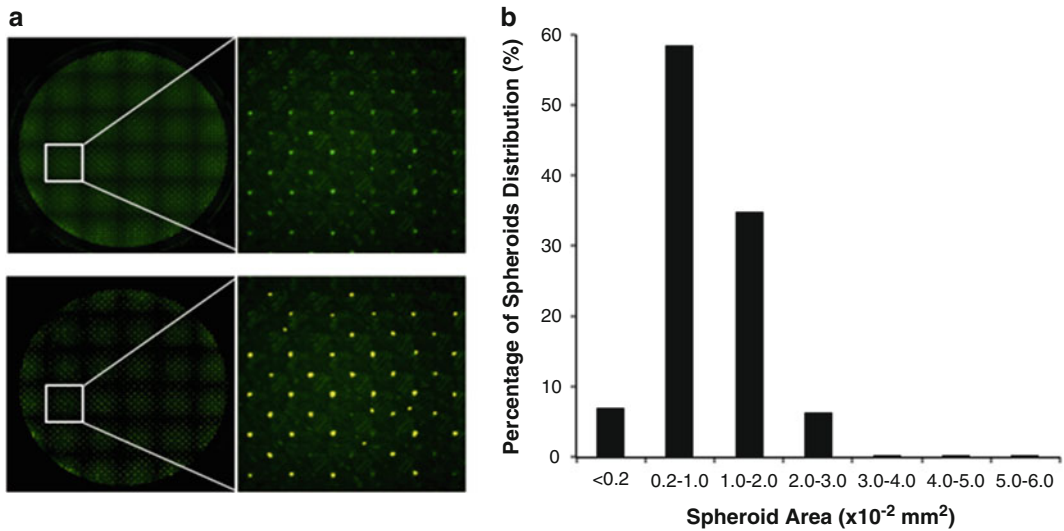


Fig. 3 Quantitative morphological evaluation of human iPSC-HE-derived spheroids through the IN Cell Analyzer 2000. **(a)** EGFP expression of human iPSC-HEs derived spheroids after 2-weeks differentiation in 3D culture (green). The lower panel shows the mask (yellow) defined as the spheroid area where the EGFP intensities are measured. The right panel is the magnification of the relative left image. **(b)** Histograms are determined by the measurement of each single spheroid area in **(a)**

specifically designed to identify spheres (i.e., bright objects) in each microsphere spot (Fig. 3a). The EGFP fluorescence intensity covers almost the entire area of the spheroids. The area of the spheroids is measured for each well (Fig. 3b). The analysis parameters are optimized for each of the assays.

4 Notes

1. Stock the solutions by aliquots at -30°C , and pre-warm at 37°C before use.
2. Be sure the solution evenly coats the dish surface; gelatin coated dish could be stored at 4°C for up to 3 days.
3. Each vial stocked should be thawed in one 100 mm dish. Remember the cell line name labeled on the vial. Minimize the amount of pipetting when thawing these cells.
4. Remember not to submerge the cap of the vial in the water bath in case of contamination.
5. Although not required, it is highly recommended that you add 10 nM ROCK Inhibitor Y-27632 to improve survival efficiency.
6. Generally, human iPSCs need to be passaged once or twice a week. We use the mechanical dissociation method to piece the

colony, followed by pretreating with disperse solution. The cells are ready for passage once they have reached about 80 % confluency in the entire dish, and half of the colonies are 1 or 2 mm in diameter. The appropriate split ratio and plating density would ensure culture cells remain in healthy and undifferentiated status before the next passage.

7. It is better to remove the differentiated colonies by pipet scraping under a microscope in sterile conditions.
8. Check cell conditions under a microscope; when the edge of colony begins to detach and the center cells still attach, stop the enzyme treatment.
9. All pipettes, tubes, dilution media, and syringes should be pre-cooled to prevent solidation of thawed Matrigel, since Matrigel rapidly forms a solid gel at room temperature.
10. Before human iPSCs differentiation, check culture cell conditions under microscope to ensure more than half of colonies are undifferentiated with healthy morphology, and do not let cells become confluent; it is crucial that they are kept as relatively small clusters that resemble the morphology of well-defined colonies.
11. Media in this stage leads to typical epithelial cell morphology change and trends to similar with definitive endoderm cells followed by differentiation, and after 6 days culture, over 80 % of cells start to express endoderm markers of FoxA2, Sox17. In FACS analysis over 60 % of cells should be CXCR4 positive.
12. Typically hepatic morphology variation has been seen after treating with FGF2 and BMP4. Continual differentiation has initiated the expression of HNF4a, followed by diminished Sox17.
13. Cell density is essential to generate uniform spheroids; 1×10^6 cells/per well in a 6-well plate is the optimized density.
14. Take care when changing media, since cell generated spheroids do not attach solidly; gently remove the old media without aspirating out cell spheroids.

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Chapter 11

The Generation and Maintenance of Rat Induced Pluripotent Stem Cells

Tomoyuki Yamaguchi, Sanae Hamanaka, and Hiromitsu Nakauchi

Abstract

This chapter describes a newly developed method for generating and maintaining rat induced pluripotent stem cells (riPSCs). We first provide a detailed protocol for the generation of lentiviral vector carrying three reprogramming factors to produce high-quality riPSCs. This technique allows reprogramming of rat somatic cells to ground state with germ-line competence. Subsequently, we elaborate a detailed protocol for the generation of riPSCs from rat embryonic fibroblast (REF). Finally, the protocols for the optimal culture conditions of riPSCs and preparation of frozen stock are described. We also outline the advantages of generating riPSCs.

Key words Rat induced pluripotent stem cells, Lentiviral vector, Dox inducible, Germ-line competent, GSK3 inhibitor, MEK inhibitor

1 Introduction

The rat has been used as an animal model to study human diseases, such as hypertension and diabetes because of the availability of many kinds of spontaneous mutants. However, because the preparation of rat pluripotent stem cells has been difficult until recently, mouse has become the leading animal for experimental genetics.

Recent advances in pluripotent stem cell research have brought about big progress towards rat experimental genetics. In 2006, Yamanaka et al. reported the generation of induced pluripotent stem cells (iPSCs) from mouse somatic cells by the enforced expression of the genes coding for four transcription factors (*Oct 4*, *Sox2*, *c-Myc*, and *Klf4*), genes that are expressed in embryonic stem cells (ESCs). Mouse iPSCs (miPSCs) are similar to mouse ESCs in terms of the expression of alkaline phosphatase (ALP), gene expression profile, chimera formation, and the ability of germ-line transmission [1, 2]. Subsequently, Ying et al. have reported generation of rat ESCs (rESCs) with use of two or three kinds of inhibitors for glycogen synthase kinase 3 (*GSK3*), mitogen-activated protein

kinase kinase (*MEK*), and fibroblast growth factor (*FGF*) receptor tyrosine kinase [3, 4]. These inhibitors have been thought to maintain a ground state of pluripotency in mESCs and can support efficient derivation and maintenance of rESCs, permitting generation of chimeric rats with germ-line transmission [5]. Shortly after the establishment of rESCs, riPSCs were successfully established with use of three kinds of inhibitors as described above [6]. We have also reported that successful generation of germ-line competent riPSCs [7]. Here we describe the detailed procedure for establishment and maintenance of riPSCs.

2 Materials

1. 0.02 % poly-L-lysine coating solution: Dilute 100 mL of poly-L-lysine solution (0.1 % (w/v) in H₂O) in 400 mL of sterilized H₂O.
2. cDMEM: Dulbecco's Modified Eagle's Medium (DMEM) with high glucose, 10 % Fetal bovine serum (FBS), 1× penicillin–streptomycin–glutamine.
3. All-in-one inducible lentiviral vector (Ai-LV): Available upon request. Helper plasmids: pCMV-VSV-G-RSV-*Rev* (RIKEN BRC), pMDLg/pRRE (Addgene).
4. 2× BES-buffered saline (2× BBS): 2.665 g BES, 4.091 g NaCl, and 2.5 mL of 150 mM Na₂HPO₄ in 250 mL solution. Adjust pH 6.95 and sterilize solution by 0.22-μm filter. Store at –20 °C.
5. 2.5 M CaCl₂: Dissolve 18.38 g of CaCl₂·2H₂O in 50 mL H₂O and sterilize solution by 0.22-μm filter. Store at –20 °C.
6. 10 mM Forskolin: Dissolve 50 mg in 12.18 mL DMSO. Store at –20 °C.
7. 0.45-μm syringe filter.
8. Phosphate-buffered saline (PBS) Ca²⁺ and Mg²⁺ free.
9. 1 mg/mL Doxycycline (Dox): Dissolve 10 mg in 10 mL H₂O. Store at –20 °C.
10. Rat leukemia inhibitory factor (10⁶ Units/mL) (rLIF, Rat ESGRO; Millipore ESG 2206).
11. 0.1 % Gelatin from porcine skin.
12. BSA Fraction V (7.5 %).
13. 0.05 % trypsin–EDTA.
14. Accutase.
15. 1 mM *MEK* inhibitor (PD0325901; Wako): Dissolve 1 mg in 2.074 mL DMSO. Store at –20 °C.
16. 3 mM *GSK3* inhibitor (CHIR99021 Axon): Dissolve 5 mg in 3.58 mL DMSO. Store at –20 °C.

17. N2B27 medium: Mix DMEM/F12 (Life Technologies) containing N2 (100×) supplement and Neurobasal medium containing B27 supplement (50×) at 1:1 ratio with 2 mM L-glutamine.
18. TC protector.

3 Methods

3.1 Generation of Ai-LV by Calcium Phosphate Transfection Method

We have successfully generated iPSCs with use of lentiviral vector which carries three mouse reprogramming factors (*Oct4*, *Klf4*, and *Sox2*) controlled by a tetracycline-responsive regulatory element (TRE) and with Ubc-promoter driven reverse tet transactivator (*rtTA*). We call this vector as all-in-one inducible lentiviral vector (Ai-LV) [8] (Fig. 1a). In comparison to several other gene delivery systems using retroviral vector [9], sendai virus vector [10] and plasmid, Ai-LV system is easy to be handled with relatively higher efficiency of reprogramming,

1. Cover the bottom of 6×10 cm dishes with a thin layer of 0.05 % poly-L-lysine coating solution (5 mL for 10 cm dish) and incubate for 10 min at room temperature. During the incubation, harvest exponentially growing 293T cells by trypsinization and count the cell number.

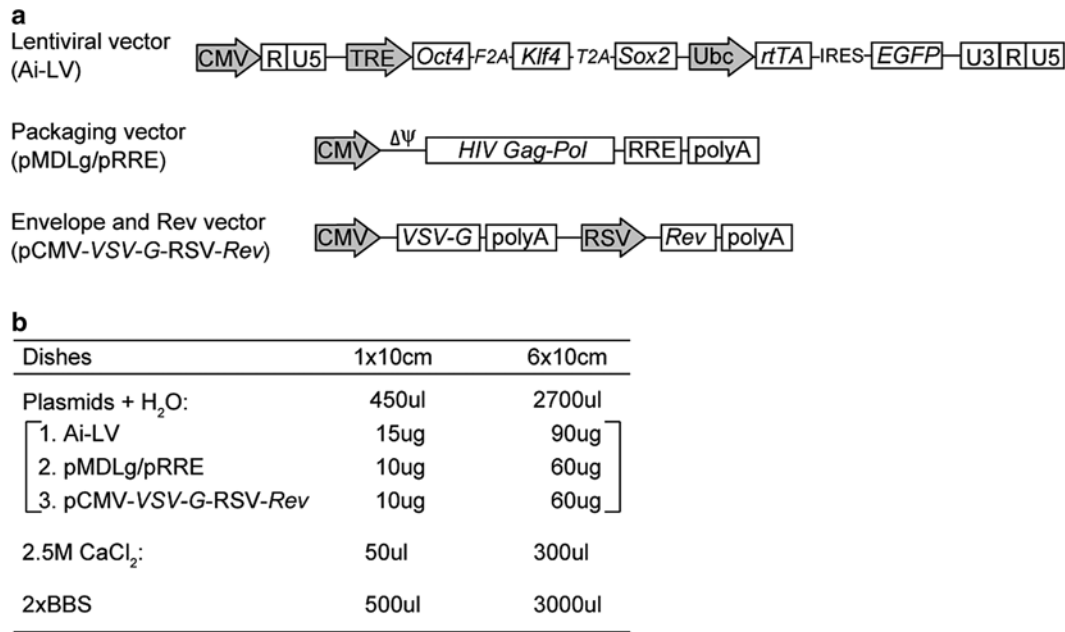


Fig. 1 (a) Schematic diagrams of all-in-one inducible lentiviral vector (Ai-LV), packaging vector (pMDLg/pRRE), and envelope and rev vector (pCMV-VSV-G-RSV-Rev). (b) The amount of components which are necessary for generation of Ai-LV (left: for 1 × 10 cm dish, right: for 6 × 10 cm dishes) by calcium phosphate transfection method

2. After incubation, aspirate off poly-L-lysine coating solution and plate 3.5×10^6 of 293T cells on coated dishes in cDMEM and incubate the cells for at 37 °C in a humid atmosphere of 10 % CO₂ for 24 h (*see Note 1*).
3. 24 h after plating the 293T cells, perform calcium phosphate transfection.
Mix 90 µg of Ai-LV, 60 µg of pCMV-VSV-G-RSV-Rev, 60 µg of pMDLg/pRRE and add H₂O up to 2.7 mL in 15 mL conical tube. Add 300 µl of 2.5 M CaCl₂ to the DNA mixture described above and vortex briefly followed by drop-wise addition of 3 mL of 2× BBS. Shake tubes vigorously and incubate the mixture for 15 min at room temperature. Drop the mixture directly to the dishes and swirl medium on the dishes (Fig. 1b).
4. Incubate the cells at 37 °C in a humid atmosphere of 3 % CO₂ for 12–16 h.
5. Aspirate off the medium and add 5 mL of cDMEM containing 10 µM Forskolin.
6. Incubate the cells at 37 °C in a humid atmosphere of 10 % CO₂ for 48 h.
7. Collect the virus supernatant and filtrate through a 0.45-µm syringe filter and transfer 30 mL of virus supernatant to 50 mL conical tube and spin at 40,000×g for 2 h at 20 °C (*see Note 2*).
8. Aspirate off the supernatant and resuspend the virus pellet in 100 µl of PBS by pipetting. Lentiviral vector suspension in aliquots can be stored at –80 °C until use (*see Note 3*).

3.1.1 Measurement of Ai-LV Titer

1. Day 0: Plate 1×10^5 of 293T cells per well of 6-well plate.
2. Day 1: Add 1, 2, 4, 8, 16 µl of concentrated virus to each well and swirl medium on the dishes. (At this point, number of 293T cells should be around 2×10^5).
3. Day 4: Analyze the ratio of GFP expressing cells (%) by FACS and calculate the infectious unit (IU).
$$\text{IU/mL} = 2 \times 10^5 (\text{cell number of 293T cells at day 1}) \times \% \text{ of GFP expressing cells} / 100 \times 1,000 / \text{volume of infected virus}$$
 (*see Note 4*).

3.2 Generation of Rat iPSCs from Rat Embryonic Fibroblast (REF)

Overview of time schedule for generation of riPSCs is described in Fig. 2b.

1. Day 0: Plate 1×10^5 rat embryonic fibroblasts (REFs: embryonic day 14.5 or 15.5) in 3 mL of cDMEM in one well of 6-well plate and incubate at 37 °C in a humid atmosphere of 10 % CO₂.
2. Day 1: Infect REFs with Ai-LV at moi = 5–10 by adding concentrated virus supernatant directly to the medium and

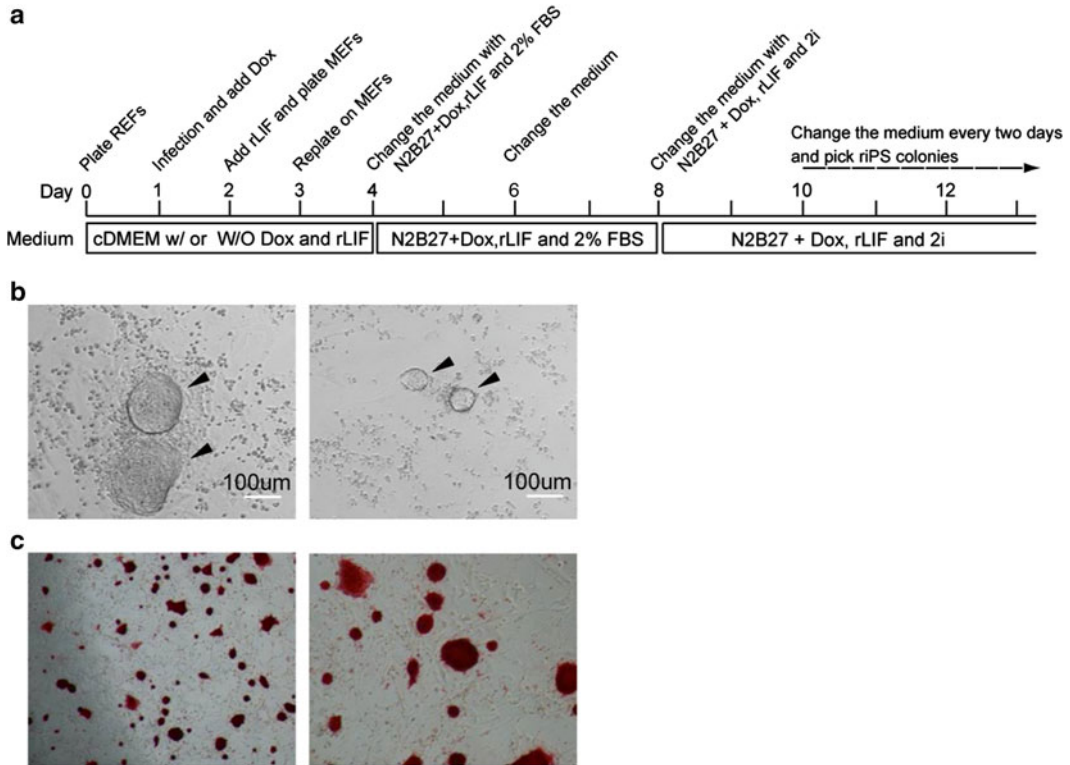


Fig. 2 (a) Schematic time schedule for generation of riPSCs. **(b)** Typical dome shape colonies of riPSCs (arrow-heads) at day 14. **(c)** Alkaline phosphatase (ALP) staining of riPS colonies at passage 15 (left: 40 $\times$, right: 100 $\times$ magnification)

incubate at 37 °C in a humid atmosphere of 10 % CO₂ for 6–8 h. Change the medium to 3 mL of cDMEM containing doxycycline (Dox) (2 µg/mL) after incubation.

3. Day 2: Add rat leukemia inhibitory factor (rLIF) (1,000 U/mL) directly into infected REF culture medium. On the same day, seed 1×10^5 mitomycin-C treated mouse embryonic fibroblasts (MEFs) in 3 mL of cDMEM in one well of 6-well plate and incubate at 37 °C in a humid atmosphere of 10 % CO₂.
4. Day 3: Harvest the infected REFs by trypsinization and plate on mitomycin-C treated MEFs which is prepared on day 2 in 3 mL of cDMEM containing Dox and rLIF at 37 °C in a humid atmosphere of 5 % CO₂.
5. Day 4: Change the medium with 3 mL of N2B27 containing Dox, rLIF, and 2 % FBS, and culture at 37 °C in a humid atmosphere of 5 % CO₂.
6. Day 6: Aspirate off the old medium and add 3 mL of fresh N2B27 containing Dox, rLIF, and 2 % FBS and continue to culture at 37 °C with atmosphere of 5 % CO₂.

7. Day 8: Change the medium with 4 mL of N2B27 containing Dox, rLIF, and 2 % FBS with PD0325901 (1 μ M) and CHIR99021 (3 μ M) (*see Note 5*).
8. Change the medium with fresh 4 mL of N2B27 containing Dox, rLIF, and 2 % FBS with PD0325901 and CHIR99021 every 2 days and morphologically ES-like colonies appear from around day 10. Select riPS colonies which show typical dome shape and pick up (Fig. 2b). Dissociate riPS colonies in accutase and plate them on mitomycin-C treated MEFs in 96-well dishes. Continue to expand riPSCs to 6-well dishes (*see Note 6*).
9. Characterization of iPS clones, (*see Note 7*).

3.3 Maintain and Freeze Stock of riPSCs

1. Unlike mouse ES/iPSC colonies, rat iPSC colonies easily come off from the bottom of dish, so that floating colonies should be collected and spun down followed by trypsinization.
2. Harvest iPS colonies attached on the bottom of dish and mix with the floating colonies described above.
3. Count the cell number and plate $3\text{--}5 \times 10^5$ cells in 1 well of 6-well dish on mitomycin-C treated MEFs in N2B27 containing PD0325901 (1 μ M), CHIR99021 (1 μ M), rLIF, and 0.075 % BSA, without Dox. Change the medium every 2 days and passage every 3–4 days (*see Note 8*).
4. Freeze and stock riPSCs: When the concentration of cell reaches 90 %, collect the iPSCs as described above and resuspend the cell pellet (1×10^6) in 500 μ l of TC protector. Freeze the cells in TC protector at -80°C overnight and then transfer to liquid nitrogen until use.
5. Thawing frozen cells: Plate the thawed cells in 1–2 wells of 6-well plate in complete growth medium (N2B27 containing PD0325901, CHIR99021, rLIF, and 0.075 % BSA, without Dox).

4 Notes

1. Poly-L-Lysine coating solution can be reused several times. Coated dishes can be stored at 4°C for a week with wrapping to prevent drying up.
2. A 50 mL conical tube which is used for virus concentration should be resistant for $4,000 \times g$. Some different methods described below can be used to concentrate virus. Low-speed centrifuge: Transfer virus supernatant to a 50 mL conical tube and centrifuge at $6,000 \times g$ for 16 h at 4°C . Resuspend the pellet in 100 μ l PBS.

Ultracentrifuge: Transfer virus supernatant to an ultracentrifuge tube (Beckman 358126) and centrifuge in Beckman SW

28 rotor with conical inserts (Beckman 358156) at $50,000\times g$ for 2 h at 20 °C. Resuspend the pellet in 100 μ l PBS.

Ultrafiltration: Transfer virus supernatant to Amicon ultra-15 (cutoff 100 kDa MW) and centrifuge $4,000\times g$ for 20 min at room temperature. This can concentrate virus 100-fold.

PEG-Precipitation: Add 1/4 volume of PEG-solution (32 % PEG6000, 0.4 M NaCl and 0.04 M HEPES and adjust pH to 7.5) in virus supernatant and centrifuge at $2,600\times g$ for 5 min at 4 °C. Resuspend the pellet in 300 μ l PBS.

3. Concentrated virus should be stored at 4 °C for short-term storage (up to 1 week) to avoid disruption of virus from repeated freeze and thaw.
4. When titrating the lentiviral vector without fluorescent marker, we determine viral RNA genome content using qRT-PCR with Lenti-X qRT-PCR titration kit according to manufacturer's protocol.
5. FGF receptor inhibitor (SU5409) in addition to two kinase inhibitors (3i) described above was used for development of rat ESCs in a previous study. However, since we found some chromosome abnormality in 3i culture, we have removed FGF receptor inhibitor (SU5409) for generation and maintenance of riPSCs. PD0325901, CHIR99021, and rLIF should be prepared immediately prior to use.
6. RiPS colonies are incubated in 20 μ l of accutase in 96-well dishes for about 10 min at 37 °C with an atmosphere of 5 % CO₂. Dissociate the colonies by pipetting and add 200 μ l of N2B27 with supplement followed by transfer on mitomycin-C treated MEFs in 96-well dishes. It is not necessary to inactivate accutase by adding medium containing FBS or inhibitor. Since all of primary colonies of riPSCs show typical dome shape and no pseudo-colonies are found, it is easy to clone riPS colonies (Fig. 2b). Moreover, all colonies show positive for alkaline phosphatase (Fig. 2c).
7. For characterization of iPS clones, we usually perform RT-PCR and immunostaining to detect pluripotent gene expression and teratoma formation and karyotyping.
8. Floating colonies resulting from mechanical shaking or rocking of dishes tend to aggregate together. To prevent the colonies coming off from the feeder layer, dishes should be treated gently.

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Protocol for Cutaneous Wound Healing Assay in a Murine Model

Gitali Ganguli-Indra

Abstract

Cutaneous wound healing assay is important to address many key questions including (1) migration ability of different cells; (2) communication between the different cell types such as keratinocytes, fibroblasts, and immune cells; (3) understanding the cell-autonomous and non-cell-autonomous function(s) of the different cells; and (4) gene regulation in healing processes. Wound healing studies can be used to test new treatment modalities, function of new drugs/compounds, and stem cell-based therapies on the different stages of healing and for accelerating wound healing in patients with compromised healing. In this chapter, we have described a simple step-by-step protocol to generate full-thickness cutaneous wounds in the dorsal skin of mice, followed by collecting the post-wounding biopsied materials on specific days for histological and immunohistochemical analyses and for RNA and protein extractions.

Key words Cutaneous wound healing, Therapeutic targets, Transcription factors, Histology, Immunohistochemistry, Protein, Biopsy, Mice, Skin

1 Introduction

Skin is the largest organ in the body and is an essential barrier with several protective functions [1–3]. Skin consists of the outer layer “epidermis” and the inner layer “dermis.” Epidermis consists of the keratinocytes which form the barrier, and the dermis consists of fibroblasts, immune cells, and endothelial cells. Skin-associated structures called “appendages” include the hair follicles, sebaceous glands, and sweat glands [4]. Therefore, it is very important that any nicks or cuts in the form of skin wounds must be efficiently repaired and the barrier restored. The process of wound healing is very complex but well coordinated and relies on right levels of growth factors, chemokines, and cytokines to successfully repair the damaged tissue [5, 6]. Wound healing is achieved through integration of four highly precise and overlapping phases: hemostasis, inflammation, proliferation, and tissue remodeling [7]. Multiple factors can cause impaired wound healing by affecting

one or more phases of the healing process. Proliferation of keratinocytes, fibroblasts, and endothelial cells help in re-epithelialization process for generating new epidermis, fibroplasia for synthesis of collagen leading to formation of granulation tissue, and sprouting of new blood vessels through the process of angiogenesis [5, 8, 9]. The different phases of wound healing involves cross talk between multiple cell types (keratinocytes, fibroblasts, endothelial cells, and different immune cells) via production of growth factors, chemokines, cytokines, extracellular matrix molecules, cell adhesion molecules, cytoskeletal proteins, proteinases, and several other factors to give rise to perfectly healed skin wounds [10, 11].

Compromised cutaneous wound healing presents significant challenges to patients and health care professionals. Therefore, full-thickness wound healing experiments are utilized to identify new therapeutic targets towards the goal of accelerating wound healing and simultaneously studying the effects of different compounds and factors on various aspects of wound healing processes [6]. Utilizing a full-thickness wound healing mouse-model, several groups have shown (1) the usefulness of different compounds such as xanthohumol, di-rhamnolipid, resveratrol, and beta-lapachone [12–15]; (2) pharmacologic activation of signaling pathways such as mammalian target of rapamycin (mTOR) [16]; and (3) use of synthetic uric acid analog [17], soluble stable and potent AngI recombinant chimera, COMP-AngI [18, 19], and different factors, to enhance wound healing process and suppress scar formation in mice with high specificity and potency [14, 15, 20–22]. In addition, gene expression profiling and network analysis studies can be done on wound healing samples taken from different days of healing, reflecting different stages, different sites within the wound bed, such as hyperproliferative epithelium, migratory tongue, and granulation tissue to identify new molecular pathways and identify genes that are common to and different between wound healing process and cancer progression. Recently, microRNA (miRNA) has gained lots of attention for their role in wound repair and as targets for therapeutic interventions in treatment of chronic wounds [23, 24].

Large number of studies have highlighted the existence of hair follicle stem cells (HFSC) and their contribution towards wound repair through cutaneous wound healing studies [4, 25–27]. Elegant lineage-tracing studies have demonstrated the role HFSC in wound repair particularly in re-epithelialization and reestablishment of skin homeostasis [4, 5, 25–31]. It is known that in response to full-thickness wounding, four types of epithelial stem cells becomes activated, exit their stem cell niches, and generate progeny that migrate out of the follicles and participate in rapid wound re-epithelialization [27–31]. But, what is not well understood is the mechanisms of recruitment of HFSC (residing in different compartments) during wound re-epithelialization, the

contribution of HFSC to different stages of wound healing, and the factors that control the migration of isthmus and bulge stem cells across these boundaries. All of these create good scopes for future studies using the cutaneous wound healing assay.

Transcription factors are known to play crucial roles in cutaneous wound repair through the modulation of cellular responses which promote the reconstruction of skin structures and functions. Role of several transcription factors in cutaneous wound healing processes has been reviewed recently [5, 8]. Transcription factors such as Smad, Stat3, homeobox proteins, hormone receptors (estrogen, androgen, glucocorticoid receptors), PPARs, API1, and SNAIL family transcription factor SLUG (*snai2*), all of which play important roles in cutaneous wound healing processes effect re-epithelialization, inflammation, and angiogenesis [32–37]. Using a full-thickness mouse wound healing assay, we have shown that transcriptional regulator chicken ovalbumin upstream promoter transcription factor (COUP-TF) interacting protein 2 (CTIP2), also known as BCL11B, has an important role to modulate cell migration, proliferation, and/or differentiation, and maintain HF stem cells during cutaneous wound healing in a cell-autonomous manner [38]. Furthermore, in a non-cell-autonomous manner Ctip2 regulates granulation tissue formation and tissue contraction during wound closure.

Use of genetically modified mice such as knockout or transgenic animal models overexpressing different key factors is one of the important tools in cutaneous wound healing research [11]. Mice has been an all-time favorite model to study wound healing mainly because of their shorter life-span, large litter size, they are less expensive, are smaller in size, and can be easily maintained and bred in the animal facility. Further advances in recent years have also seen the use of constitutive and inducible transgenic mice along with tissue specificity and lineage-tracing studies to elucidate the contribution of different factors and cell types during wound healing processes [27, 29, 38, 39]. The complexity and coordination of the healing processes are major hurdles for therapeutic intervention due to lack of proper alignment between a given therapeutic agent and a particular stage of healing is essential. Therefore, tremendous efforts are on for better understanding of the different stages of the wound healing process and elucidation of the underlying mechanisms, thus making wound healing studies a top priority.

2 Materials

1. Biosafety hoods.
2. Small animal anesthesia system or anesthetic ketamine/xylazine.
3. Clipper for shaving.

4. 70 % Ethanol.
5. Fine tissue scissors and forceps, gloves (surgical grades).
6. High-resolution digital camera.
7. Any image analysis software such as Photoshop or Image J.
8. Standard supplies required for paraffin embedding of the biopsied tissue samples, followed by sectioning and immunohistochemistry.
9. RNA later for preserving the tissue for RNA extraction to be processed later.
10. Liquid nitrogen for freezing the tissue sample if needed for protein extraction.

3 Methods

3.1 Generation of Full-Thickness Wounds in Mice Must be Carried Out in Standard Animal Facility

1. Mice that will be used for the study must be kept in the standard housing conditions at the satellite animal facility. Animal protocol must be approved by the Institutional Animal Care and Use Committee (IACUC).
2. 6–8-week-old mice can be used for the study. These mice have to be anesthetized using any traditional isoflurane-based small animal anesthesia unit (Fig. 1a, b).
3. After anesthetizing the mice, shave the dorsal side of the mice with electric clippers (Fig. 1c). Wipe the area with 70 % ethanol and allow it to dry.
4. Generate 2–4 full-thickness excisional wounds (5 mm in size) on the back skin of anesthetized mice using sterile biopsy punches (*see Note 1*). Briefly, you must hold the skin from the mid dorsal line and push the biopsy punch into the skin folds with a twisting motion left and right and punch out both the layers (Fig. 1d, e). This is the easiest way for you to generate two punches on each side of the mid dorsal line (Fig. 1f). Using the surgical forceps remove the circle piece of skin (Fig. 1g). The day of the punch biopsy is considered as day 0.
5. Photographs are taken using a digital camera each day after the biopsy. Mice have to be placed uniformly every day for photography (*see Note 2*). Keep the camera settings and height of the camera fixed (*see Note 3*). Wounds must be photographed and measured again on specific days according to experimental plans (*see Note 4*). The diameter of each wound is then measured using Photoshop (version 6.0; Adobe system, San Jose, CA) to calculate the percent of wound closure compared to day-0 wound diameter. Healing can be defined as the decrease in wound diameter over time and was expressed as the percentage of the day-0 wound diameter [38].

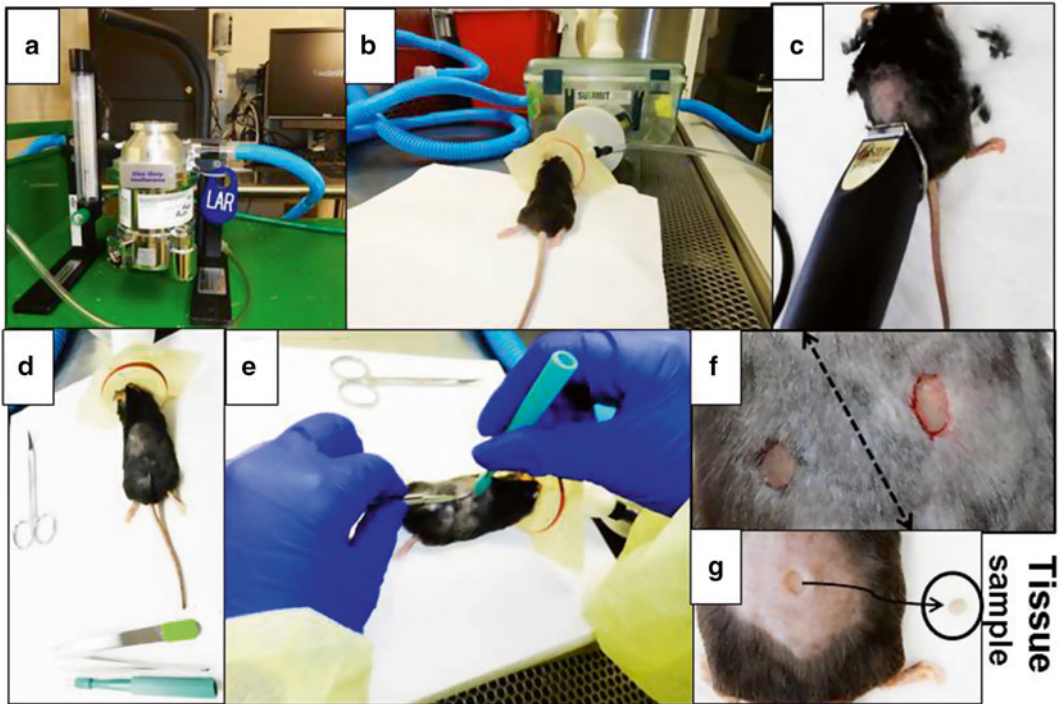


Fig. 1 Routine setup for generating full-thickness wounds in mice. (a, b) Small animal anesthesia system in use. (c) Shaving is done with a clipper prior to inflicting wounds. (d) Mice is now ready for the punches. The procedure needs good-quality scissors, forceps, and sterile punches of fixed sizes (5 mm). (e) Generation of full-thickness wound with the biopsy punch. Either one or two wounds can be generated. (f) Equal-size two wounds can be created by holding the mid dorsal skin and pressing the biopsy punch into both sides of the skin folds followed by punching out both the layers. (g) The punch biopsy tissue sample which can be cut into two halves and used for different assays

6. These mice must be monitored daily including weekend and holidays until the wound closure is achieved (*see Note 5*).
7. Samples from different time points of healing can be chosen that covers the specific stages such as hemostasis (6–12 h), inflammation (1–3 days), proliferation (5–7 days), and remodeling (12 days). Representative groups of mice at different time points after wounding/injury are euthanatized and wound tissue including 5 mm surrounding wound margin skin is harvested. Briefly, excise the wound completely with approximately 5 mm border around the wound. Place the piece of skin flat (dermis side down) on a piece of Whatman filter paper and bisect into two halves, one half immediately frozen for protein extraction and the other in suitable fixative for histopathological analysis (*see Note 6*).
8. Follow standard protocol for fixation and paraffin embedding of the tissue sample (*see Note 7*) [38]. The samples to be used for RNA should be stored in RNA later (*see Note 8*).

Samples for protein extraction can be snap frozen in liquid nitrogen and then stored in -80°C freezer until use (follow extraction procedure depending on the assay you wanted to perform).

4 Notes

1. Use of the fixed sized punches will ensure uniform size wound generation and therefore minimizing errors in the wound closure analysis.
2. Align a metric ruler next to the wound for size references which will aid in the measurement of the wound diameter.
3. Better to use a camera with a stand so that the height of the camera is fixed for all the experimental days.
4. One of the important factors which affects wound healing is stress response that can occur during the entire process of handling and photography times, therefore minimizing these times.
5. Be vigilant for any signs of pain and discomfort. Maintain these mice in ventilated racks or micro-isolators to avoid any kind of secondary infections.
6. For most of the histology applications, we use 4 % paraformaldehyde in PBS for fixation. We make several batches of 4 % paraformaldehyde in PBS, store frozen in -20°C , and thaw them at the time of use. Tissue can be fixed in either freshly made or frozen thawed fixative.
7. We normally embed the tissue samples in fixative, follow a standard protocol for fixation, and embed on a tissue embedding station which is designed for embedding tissue in paraffin (Fig. 2a–d). Paraffin sections are then cut using manual rotary microtome (Fig. 2c and the inset). We routinely perform hematoxylin and eosin staining (H&E stain) on these sections as well as perform immunohistochemistry with antibody specific to markers of interest.
8. Samples must be about 0.5 cm thickness in any one dimension and in 5 volumes of solution as recommended by the manufacturer and stored at 4°C for 1 month or more (TRIzol[®] Reagent for RNA extraction can be used).

5 Conclusion

Murine excision wound healing is an ideal model for elucidating the role of growth factors, inflammatory cytokines, and chemokines in wound healing processes. These studies can lead to identification of new targets, and novel drugs/compounds for more

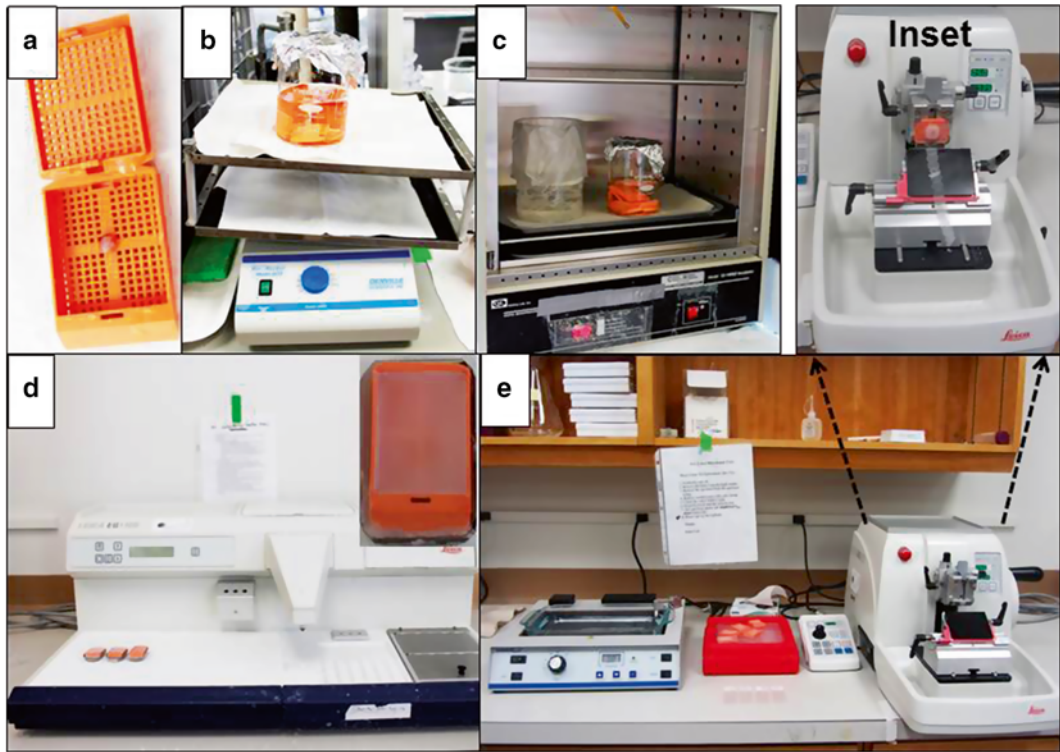


Fig. 2 Routine histological procedures for processing of the wound biopsies. (a) Tissue sample is captured directly in the tissue embedding cassettes and routine procedure for paraffin embedding of tissue samples is carried out, which includes fixation, dehydration, clearing (b, c), and paraffin embedding (d, e, and the *inset*) using the standard tissue embedding station. Paraffin sections are then cut on a manual rotary microtome (e and the *inset*) and used for routine H&E staining and immunohistochemical assays

efficient treatment and healing of otherwise chronic wounds, burn wounds in military individuals, and wounds found in people with underlying diseases.

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Chapter 13

Adipose-Derived Stem Cells: Methods for Isolation and Applications for Clinical Use

Brian Mailey, Ava Hosseini, Jennifer Baker, Adam Young, Zeni Alfonso, Kevin Hicok, Anne M. Wallace, and Steven R. Cohen

Abstract

Adipose tissue sciences have rapidly expanded since the identification of regenerative cells contained within the stromal vascular fraction (SVF) of fat. Isolation of the SVF, containing adipose-derived stem cells (ADSC), can be accomplished efficiently in the operating room or in the laboratory through enzymatic digestion of the adipose tissue and concentration of SVF. Cells can be directly re-injected as a mesotherapeutic agent, recombined with a tissue scaffold (e.g., cell-enriched fat grafts) or expanded in culture for tissue-engineered cell therapeutics. The potential for cell therapy is under current investigation by researchers around the world. This chapter reviews laboratory methods for isolating ADSCs and the ongoing clinical trials evaluating cell therapeutic efficacy across many specialties, including cardiology, neurology, immunology, tissue engineering, sports medicine, and plastic and reconstructive surgery.

Key words Adipose-derived stem or regenerative cells, Isolation, Laboratory methods, Clinical trials

1 Introduction

1.1 *Developments in Adipose Tissue Sciences*

The detection of endogenous stem cells in human fat [1, 2] has drawn attention to the fields of tissue engineering, regenerative medicine, and developmental biology. This discovery creates new promise and prospective treatment strategies for chronic and degenerative disease, including arthritis, heart disease, autoimmune disorders, and potentially organ regeneration [3, 4]. Resultant, for plastic surgery, has been a surge in popularity for fat grafting procedures to replace lost tissue volume [5, 6]. Furthermore, adipose tissue sciences have become a funding priority for various organizations and fundamental, developmental programs. Interest is further propelled by the abundance, availability, and accessibility of fat, which obviates one of the most difficult problems in reconstructive surgery: using expendable tissue to heal wounds, fill gaps, and regenerate tissue.

The popularity of the liposuction technique rose in the 1980s to become the most common plastic surgery procedure performed today. This, in turn, led to the modern era of fat grafting, which now ranks ninth among the most popular surgical cosmetic procedures [7]. In the past, adipose tissue was treated as inert tissue primarily serving an energy storage function. Over the past decade, research has revealed the diversity and potential of cells contained within the seemingly homogenous fat tissue, which is now believed to play many roles in maintaining homeostasis and self-repair [7, 8]. Utilizing adipose tissue and its accompanying stromal vascular fraction (SVF), which includes adipose-derived stem cells (ADSCs), has become an office-based, point-of-care technique efficiently accomplished by individual practitioners using manual or semiautomated proteolytic tissue processing systems. In this chapter, we discuss the current technologies available for isolating SVF and ADSCs and clinical applications for their use.

1.2 Composition of Adipose Tissue

In the adult, fat comprises mainly white adipose tissue, which is located under the skin, around thoracic and abdominal organs, and in the perivascular space encasing blood vessels. The majority of adipose tissue is composed of mature fat cells called adipocytes, which store triglycerides in large lipid vacuoles [7]. When removed from the body, adipose tissue can readily be enzymatically digested by collagenase, releasing two fractions: a layer of mature adipocytes, which rise to the surface, and a non-buoyant SVF consisting of leukocytes, fibroblasts, erythrocytes, vascular-associated cells, and multipotent ADSCs [9]. Two of the required characteristics for a cell to earn the “stem cell” classification include the capacity for multipotent differentiation and clonogenicity [1]. ADSCs behave similarly to bone marrow-derived mesenchymal stem cells [10], exhibiting multipotent plasticity, possessing the ability to differentiate towards several cell types including osteoblasts, chondrocytes, adipocytes, and myocytes [2, 11, 12]. Some studies have also proposed an ability of these cells to espouse complex visceral tissue, like heart, liver, and brain [13–15]; these claims remain debated. Telomere lengths in ADSC chromosomes have been used as an explanation for differences in activity and behavior of cells from varying individuals, but the presence and activity of telomerase within ADSCs remain uncertain. Because the cells exhibit some degree of senescence in vitro, their progenitor status has been questioned [16, 17]. However, the multipotency observed in culture and upregulation in gene expression of stem cell markers, such as OCT4 and SOX2, have justified ADSC classification as adult stem cells [16, 17].

1.3 Growth Factors

Stem cells are designed to construct and maintain homeostasis, through mechanisms such as cellular replacement [8]. If a stem cell is grafted into a host environment, a dynamic relationship occurs. The host signals and directs the stem cell towards differentiation

and, in turn, the cell signals back to the host. This interaction governs neovascularization, regulation of apoptosis, modulation of immune response, and extracellular matrix remodeling, which is mediated through growth factors, cytokines, and extracellular matrix molecules [18] [19]. Adipose tissue is known to produce remarkable amounts of growth factors. Analysis by enzyme-linked immunosorbent assay (ELISA) demonstrates significant levels of bFGF, IGF-1, VEGF, and PDGF-BB contained in routine lipoaspirate [9, 12]. The secretion of these signaling molecules is considered a likely explanation for regenerative and tissue remodeling properties of fat grafts. Clinically this has been demonstrated through improvements in skin elasticity damaged by irradiation and improvements in dyschromias and skin pigmentation after cell-enriched fat grafting for aesthetic patients [20]. De novo or increased angiogenesis is also promoted by growth factors, with VEGF being considered one of the most relevant. The governance of cellular differentiation and adipose tissue proliferation is also attributed to growth factor secretion [21].

The constantly evolving relationship between host microenvironment and therapeutic cells makes cellular therapy challenging to manipulate, as disease is not driven by a single pathophysiological process. In amyotrophic lateral sclerosis (*aka*, Lou Gehrig's disease), for instance, a fatal neurodegenerative disease affecting motor neurons, animal studies reveal that the pathologic process involves more than simply motor cell death and therefore, successful treatment involves targeting multiple cell types. Experimental outcomes from cells implanted into the central canal of murine spinal cords demonstrate delay in motor impairment. Subsequent histological analysis revealed neuron replacement (i.e., increased number of motor neurons), but also neuroprotection with sparing of existing motor neurons, decreased inflammation, trophic support, and intracellular mitochondrial mediated homeostasis [22]. Directing stem cell grafts to regenerate or repair damaged tissue is dependent on host-cell communications. In the neuroimmune system, a neural stem cell gives rise to a neuron, which signals inhibition of neuronal growth and release of neurotrophic, neuroregulatory (e.g., GDNF) factors activating glial cells to chaperone the neuron [23]. Alternatively, the cellular signaling may theoretically be damaging by production of a deleterious protein or promoting unwanted angiogenesis in tumor formation. Experimental in vitro and in vivo reports on rodent studies report osteosarcoma recurrence after lipofilling [24]. In vitro experiments with ADSCs and tumor cells led to enhanced tumor growth, an observation confirmed by similar tumor growth response in mice after co-injection of tumor cells and ADSCs [25]. However, the spontaneous malignant transformation of ADSCs has not been seen in animals or humans. Furthermore, a number of clinical reports reviewing the incidence of breast cancer treated with lipofilling have not reflected any additional cancerogenic effects [26].

1.4 Tissue Engineering

Tissue engineering aims at developing techniques for using autologous or allogeneic cells for treating damaged or lost tissues. For visceral tissue, the extracellular matrix (ECM) provides form and structure to the organ. This biological matrix can be transferred between organs, and after a period of inflammation, the implanted scaffold transforms into normal tissue resembling its host. This process occurs as the ECM provides an “information highway” allowing intercellular communication and recruiting multipotent cells to differentiate into tissue-specific cells [27, 28]. In vivo evidence for this mechanism is provided through analysis of the ECM infiltrate in an amputated murine hind foot model. Specific mononuclear cells of the infiltrate can differentiate into almost any tissue type, depending on the ECM medium in which the cells are grown, suggesting that multipotent cells are recruited to the site of injury by a chemotactic mechanism inside the ECM [29]. In a canine model studying musculotendinous regeneration, after resection of a portion of Achilles tendon and gastrocnemius muscle, a scaffold created from porcine small intestine submucosa was implanted. Histologic analysis at 90 days showed no difference in cellularity from the normal Achilles tendon [30]. For adipose tissue engineering, the transplantation of essentially unaltered fat as a natural filler from one area of the body to another to correct volume deficiencies and alter contour deformities is in wide clinical practice around the world. However, the specific regenerative capacities of the adipose cellular component are still currently being investigated. Two general approaches are being used: (1) in vitro seeding of biomaterials with ADSCs to act as a delivery system and (2) the recruitment of in vivo endogenous tissue as a native matrix.

In vitro seeding accomplishes ADSC delivery by using a biomaterial as a scaffold. Successful seeding in vitro can be achieved using publically available, validated laboratory protocols. The SVF is first released from adipose tissue mechanically or enzymatically. Enrichment of ADSC populations can then be accomplished by expanding the cells under specific cell culture conditions, which allows differentiation into various cell lineages. For example, addition of growth factors, namely VEGF or IGF-1, stimulates conversion of naïve cells into mature tissue types. It has also been demonstrated that ADSC differentiation may be improved by replacement of fetal calf serum with human serum during in vitro cell cultivation studies [31]. Finally, ADSCs are seeded in a scaffold that houses the cells and allows exchange of soluble nutrients and secreted molecules. The implant should be biocompatible, without eliciting a significant inflammatory response either from the donor or from the transplanted cells. Current research focuses on the combination of engineered extracellular matrices and cultured cells. Success is limited by mechanical stability, constricted adipose tissue volume, cell differentiation, and insufficient nutrition and oxygen supply.

Current applications of these in vitro adipose tissue scaffolds are for the treatment of small volume defects, while treatment of larger wounds cannot yet be effectively accomplished by this technology.

In vivo tissue engineering uses the body's ability to repair by generating its own biological scaffold. Various medications, including the antipsychotics haloperidol or clozapine or proinflammatory mediators, like chemokine ligand 2 and monocyte chemoattractant protein-1 (MCP-1), induce massive weight gain by adipogenesis [32, 33]. Mechanistically, the transmigration of monocytes out of the blood into peripheral tissues stimulates differentiation and proliferation of ADSCs to form mature fat tissue [34]. These pharmacologic induction agents of cellular proliferation are currently under investigation. An alternative concept expands an endogenous ECM with the external tissue expansion BRAVA system (Brava LLC, Miami, FL) [35, 36]. This externally applied vacuum induces cell proliferation and differentiation resulting in the formation of an autologous breast scaffold, which is filled by serial fat grafting [35, 36]. This method is touted as "incisionless" breast reconstruction or augmentation and takes 6 and 13 months for total breast creation in non-radiated and radiated patients, respectively. The expansion is accompanied with a mean of 2.8 (non-radiated) and 4.7 (radiated) fat grafting sessions.

Clinical trials are under way to evaluate outcome measures of tissue grafts such as biosafety, biocompatibility, graft durability, and clinical outcomes (e.g., patient survival, graft patency, and quality of life). A trial using cadaveric tracheas for biologic scaffolds demonstrated completely differentiated respiratory epithelium at 2–6 months post-transplantation [37]. However, artificial tissue-engineered scaffolds that mimic the bioproperties of native tissue may be a more effective "off-the-shelf" alternative, as wait times and degradation/stenosis rates are less than the cadaveric approach [38].

1.5 Ongoing Research in Adipose Tissue Sciences

Advancements in adipose-based stem cell therapies are being driven both by basic and clinical research. The traditional bench to bedside process, characteristic of traditional pharmaceuticals, can be accelerated or bypassed for adipose-based therapeutics, due to the inherent autologous nature of this therapy and abundance and expendability of fat. To date, cell-based therapies are under examination by the US Food and Drug Administration, the institution that regulates the safety of biological products. Specifically, the Center for Biologics Evaluation and Research (CBER) has deemed autologous cells to be "minimally manipulated" and therefore not subject to the quality control regulations associated with synthetic drug production. Independent task forces developed under the National Institutes of Health and a separate joint task force of the American Society of Plastic Surgeons and the American Society for Aesthetic Plastic Surgery were created to investigate the scope of treatments and available scientific evidence to use stem cells in clinical practice.

Many other parts of the world actively use cell-enriched therapy for clinical and research purposes. The best practices of cell therapies, and major obstacles preventing further clinical application, are being concurrently studied across multiple disciplines, including cardiovascular diseases, neurological and immune conditions, reconstructive and aesthetic plastic surgery, sports medicine, and tissue engineering.

1.5.1 Cardiology Applications

Current clinical trials in cardiology include stem cell therapy for heart failure, angina, and cardiac transplantation. In the ongoing ATHENA multicenter, double-blinded, randomized, placebo-controlled trial, patients with heart failure from coronary artery disease received intramyocardial injection of ADSCs ($N=30$) versus placebo ($N=15$) to confirm safety and feasibility of intracardiac stem cell delivery. The safety and efficacy of autologous bone marrow mononuclear cell therapy have already been reported in the Texas Heart Institute's FOCUS-HF trial [39], comparing cell treated ($n=20$) to control ($n=10$) no option (i.e., not candidates for other standard therapies) patients. The authors of this trial also reported improved functional capacity in the cell-treated group and a variability of potency in stem cell quality, with the stem cells of younger patients being more efficacious than their older counterparts [39]. This finding has been observed by others and explained by changes in telomere length [4, 40]. The FOCUS trial is a follow-up to its predecessor evaluating the primary outcomes of myocardial perfusion, left ventricular contractile performance, and maximal oxygen consumption in 86 patients with reversible ischemia. Preliminary studies show significant improvements in ejection fraction [41]. The PRECISE trial further corroborated the safety of adipose-derived regenerative cell administration by showing similar rates of major adverse events and mortality between the recipient group ($n=21$) as compared to controls ($n=6$) in patients with ischemic heart disease. In the treatment of refractory angina, the MHI OPTIMIST (Minneapolis Heart Institute, OPTions In Myocardial Ischemic Syndrome Therapy) program [22], a registry of 1,400 patients with refractory angina, is evaluating the angiogenic potential of stem cell therapies whereas other studies using autologous intramyocardial CD34⁺ stem cells have shown a reduction in ischemic zones, improvement in exercise time, improved quality of life, and reduction in mortality in cell-treated patients [42]. Stem cell-assisted therapy is also being considered for development in cardiac transplantation. Research is not as advanced for this modality as in heart failure and ischemic heart disease; however bioprinting and decellularized organ scaffolds are in early stages of investigations and may have future clinical applications.

1.5.2 Immunology Applications

The use of SVF as a mesotherapeutic agent for various autoimmune disorders is being evaluated in clinical trials. In a study treating patients with scleroderma, a mean of 50 million cells isolated from 174 mL of fat was injected into patient hands with a resultant 55 % improvement in Raynaud's severity and 52 % improvement in health assessment. At Northwestern University in Chicago, over 120 patients with multiple sclerosis (MS) have been treated with intravenous hematopoietic stem cell therapy in the first study to show the reversal of disability in MS patients. This effect may be due to stem cells' influence on the immune system. Specifically, stem cells suppress T and B cells, thereby arresting cell proliferation, and impair the maturation of dendritic cells leading to the inability of these cells to present antigens. Mesenchymal stem cell (MSC)-treated patients had decreased number of antibodies against myelin, clinically resulting in decreased demyelination in MS. Additional in vivo studies demonstrate that MSCs do not differentiate into neurons as originally thought, but instead protect already existing neurons through substances they secrete. Proposed mechanisms leading to neural protection include prevention of oxidative stress and reduction of microglial activation. Activated microglia led to upregulation of pro-inflammatory molecules and downregulation of receptors associated with neuronal protection. This response is hindered by injection of MSCs [43]. In Crohn's disease, ADSC injection has been successful in anal fistula closure.

1.5.3 Plastic and Reconstructive Surgery Applications

Plastic surgeons have frequently been at the forefront of surgical innovation and transplantation. The noble laureate and surgical innovator, Joseph Murray, was the first to perform a renal transplant between living identical twins [44]. Forty years later, plastic surgeons were pioneers in composite tissue allografts performing full transfers of a human face [45]. Plastic surgeons popularized liposuction in the 1980s and noticed early on the regenerative and multipotent stem cells in fat. These therapies are now being studied for children with craniofacial anomalies and microfacial hemi-somnia, alopecia, skin dyschromias, and breast mound creation after mastectomy [6, 20, 35, 46, 47]. Successful applications for facial fat grafting have emerged, especially with the addition of platelet-rich plasma (PRP) and/or SVF to traditional fat graft samples [48, 49]. Facial fat grafting has also been used in treating HIV patients with facial lipoatrophy, seen as a side effect from protease inhibitors. Both SVF- and ADSC-enriched fat grafts demonstrated higher volume retention than traditional fat grafts a year-and-a-half post-grafting [50].

1.5.4 Neurology Applications

The therapeutic and immunomodulatory activities of MSCs are being examined in the treatment of various neurologic disorders, including stroke, Parkinson's disease, and Huntington's disease. Ischemic stroke remains one the leading causes of death and disability

in industrialized nations; however, current therapy remains limited. While the use of thrombolytic therapy with alteplase has improved clinical outcomes, its efficacy is restricted to a narrow time window of hours after symptom onset and is not appropriate for all patients [51]. Cell therapy for treatment in acute stroke has shown promise in animal and early clinical studies. Murine studies demonstrate neural protection with bone marrow-derived mononuclear cells (MNCs). When mice are randomized to get graded doses of MNCs or saline, animals receiving higher doses have better outcomes than mice receiving smaller doses or placebo [52]. In vitro studies suggest that the therapeutic mechanism may be through modulation of the neurotoxic response of microglia after a stroke. Additionally, when activated microglial cells are first treated with MNCs, there is an increase in neuronal viability. There is also an increase in vessel density suggesting increased angiogenesis [53]. The safety of intravenously administered MNCs for acute stroke (SIVMAS) is a phase I study with 10 patients, later expanded to 25, who underwent iliac crest bone marrow harvest within 24–72 h of stroke symptoms. The MNCs were separated and then re-injected into patients. The procedure was successfully performed in all patients with no adverse events related to infusion. Although there were no control patients for this pilot study, an age-matched cohort from the stroke registry suggests that patients who received MNCs have a disability linear trajectory similar to controls [54]. Future randomized trials will be directed towards evaluating efficacy as a primary endpoint. While the role of MSCs in stroke has been investigated using bone marrow MSCs, the high accessibility and ease of harvest of adipose-derived MSCs make this acquisition source particularly attractive and recent studies show promise that they may be at least as effective. Importantly, when cultured under appropriate conditions, no phenotypic differences in antigen markers between MSCs isolated from adipose tissue, human bone marrow, or umbilical cord blood were observed by flow cytometry using a panel of 22 surface antigen markers [55]. The superiority of adipocyte-derived MSCs over bone marrow MSCs was demonstrated in an animal model of liver injury [56]. A recent rat model also demonstrated significantly limited brain infarction area and improved sensorimotor dysfunction with IV adipose-derived MSC administration after ischemic stroke [57]. Beyond cell suspensions, solid tissue graft transplantation is also being studied in neurologic diseases such as Parkinson's (PD) and Huntington's (HD). In PD patients, post-transplant FD-PET studies show increased dopamine levels and autopsy of the first grafted patient 18 months after transplantation revealed by histology both graft survival and integration of transplanted tissue into recipient brain tissue. However, in patients surviving 14 years after transplant, grafted neurons showed Lewy bodies on histology and progression of Lewy body development in grafted neurons mirrored the natural progression of PD but on an accelerated time course (i.e., no alpha-synuclein seen at 18 months,

cytoplasmic alpha-synuclein at 4 years, and aggregated deposition at 14 years). Despite the eventual development of PD in these transplanted grafts, patients had significant clinical improvement with relief from dyskinesias. Interestingly these improvements were most pronounced while graft patients were receiving immunosuppression and clinical deterioration was noted when immunosuppression was stopped, suggesting that long-term immunosuppression may be necessary and also that inflammation may play a critical role in the development of PD in these cells [58]. Of note, it has been recently shown that inflammation in cell suspension transplants is lower as compared to solid tissue transplants and these cell suspension grafts have a lower percentage of neurons with Lewy bodies as compared to solid grafts, supporting this theory. In contrast to the promising results in PD patients, tissue transplantation in HD patients has not found similar early success. HD patients with tissue transplantation showed minimal clinical improvement over a shorter period of time, and, due to the increased morbidity associated with surgery in HD patients, risks currently outweigh benefits in further exploring tissue transplantation in this population [59].

1.5.5 Tissue Engineering Applications

The goal of tissue engineering is to create nonimmunogenic functional tissue replacements. Use of decellularized scaffolds, which become autointegrated via cell migration, provides a possible technique for organ regeneration. In an 80-patient multicenter clinical trial, patients with 50 % or greater loss of mass of a specific muscle group were enrolled to undergo surgery with scar excision and implantation of an ECM scaffold. On postoperative day 1, treated patients started a customized weight-bearing physical therapy regimen so that the cells being recruited into this scaffold receive the signal to become muscle. Without the signal to bear weight, these cells could potentially differentiate into connective tissue. Postoperative biopsy revealed mononuclear infiltrate at 6 weeks and skeletal muscle at 6 months. All patients reported improvement in quality of life, with the majority showing successful functional outcomes.

1.5.6 Muscle Injuries and Disorders

Up to 55 % of all injuries sustained are muscle related. Current treatments are conservative and have not changed over the years, but new data supports stem cells as a potential new treatment. In animal studies, rats with muscle injury treated with ADSC injection showed increased tetanic force and increased myofiber regeneration at 2 weeks [60]. In muscular dystrophy (MD), more recent trials favor a “stem cell exhaustion” theory as the cause of symptoms. Early in life, stem cells are able to repair injured muscle and therefore a patient is asymptomatic. As stem cells are depleted with time, however, and as “stem cell exhaustion” is reached, the stem cells cannot keep up with muscle damage and symptoms appear [61]. Steroid treatment in MD patients has increased

patient life-span and delayed muscle loss, likely by reducing stem cell exhaustion. In studying muscle and bony injuries, PRP, microfracture, and BMAC (bone marrow aspirate concentrate) may speed the healing process. The largest clinical experience has been with PRP, which may improve healing through multiple mechanisms including increased angiogenesis, enhanced collagen synthesis, and proliferation of cell types that promote epithelialization and granulation tissue production [62]. Case series of professional athletes treated with adjunct PRP suggest that PRP therapy may promote faster recovery times with return to maximal performance at earlier time points. Using PRP in combination with microfracture (creating an environment where blood flows to a surface defect, creating a platelet plug) may lead to synergistic healing. BMAC (bone marrow aspirate concentrate) has been shown to improve pain and range of motion. Therefore, BMAC-combined treatments may also have potentiating effects [63].

1.5.7 Conclusions

ADSCs have received substantially increasing attention over the past decade, both in the laboratory and clinic. Excitement for fat-derived cell therapies is based upon four key factors: (1) their ease of harvest by routine liposuction techniques, (2) the multipotency making them ideal candidates for many experimental treatments, (3) ease and efficiency of isolation with sterile techniques, and (4) the immunoprivileged status inherent in their autologous nature. Current clinical trials have yielded exciting results to date across specialties, including treatments for heart failure, multiple sclerosis, sports-related injuries, radiation skin damage, and many more. Accordingly, ADSCs have a promising future that is deserving of further clinical attention and research funding.

2 Materials

2.1 Liposuction (per 1 L Normal Saline)

1. 25 mL 1 % lidocaine.
2. 1 ampule 1:1,000 epinephrine.
3. Liposuction system.
4. Fat filtration system (e.g., PureGraft) (optional).

2.2 Adipocyte Digestion and Isolation of SVF

1. 0.075 % Collagenase I (or Celase®): Dissolve 100 mg Collagenase type I in 132 mL of DMEM and 1.3 mL 10,000 IU penicillin/10,000 µg/mL streptomycin to obtain 0.075 % Collagenase I (1 % P/S). Sterile filter through a 150 mL 0.22 µm filter, aliquot 4 mL per 15 mL conical tube, and store at -20 °C until use.
2. 160 mM NH₄Cl: Dissolve 427.9 mg NH₄Cl in 50 mL dH₂O (or 8.55 g in 1 L dH₂O). Sterile filter through a 50 mL 0.22 µm filter, aliquot 4 mL per 15 mL conical tube, and store at -20 °C until use.

3. *h*ASC growth media: Remove 52.5 mL from a new 500 mL DMEM/F12 bottle. Add 50 mL FBS (10 %) and 2.5 mL of 10,000 IU penicillin/10,000 µg/mL streptomycin (1 %). Sterile filter through a 1 L 0.22 µm vacuum filtration unit. Store at 4 °C.
4. DMEM buffer.
5. 2 % bovine serum albumin.
6. FACS Lysis buffer.
7. T-75 (75 cm²) tissue culture flasks.
8. 1 % penicillin/streptomycin.
9. dH₂O.

3 Methods

3.1 Clinical Applications: Fat Harvest, Preparation, and Grafting

Retrieval of SVF is most commonly accomplished from liposuctioned fat or other discarded sources of adipose tissue, such as abdominoplasty or direct lipectomy (*see* **Notes 1** and **2**). These methods generate an abundant available source of cells from which to derive ADSCs [10]. Various liposuction techniques and devices are currently used. However, they generally all incorporate some method of fluid tumescence (including 0.1 % lidocaine and 1:1,000,000 epinephrine—to limit blood loss), followed by a negative suction force to extract the lipoaspirate. Fat can be collected sterilely, if needed for re-injection, or off the table in non-sterile conditions for other purposes (*see* **Note 3**).

1. Our routine consists of combining 25 mL of 1 % lidocaine with 1 mL of 1:1,000 epinephrine into each liter of saline infused.
2. The tumescent is allowed to sit for 10–15 min and then fat is aspirated using 3–4.8 mm cannulae (*see* **Note 4**).
3. Fat for injection is prepared by either (1) allowing it to separate by gravity and (2) centrifugation or (3) washing the fat with lactated Ringer's using the PureGraft system. If the former or the latter method is used (usually for breast or body fat grafting), the fat is re-collected with 60 mL syringes, transferred to 10 mL syringes, and injected with blunt-tipped cannulas (Fig. 1) (*see* **Note 5**).
4. Smaller volumes of fat are usually harvested for facial fat grafting. In these situations, fat is centrifuged at 3,000 rpm for 3 min in 10 mL syringes and transferred to 1 mL syringes for injection (*see* **Note 6**).

3.2 Laboratory Techniques: Isolation of the Stromal Vascular Fraction

1. Freshly obtained lipoaspirate or raw fat is washed with lactated Ringer's solution to remove contaminating blood and remaining tumescent solution. The non-buoyant nucleated cells, commonly referred to as the SVF, are proteolytically released

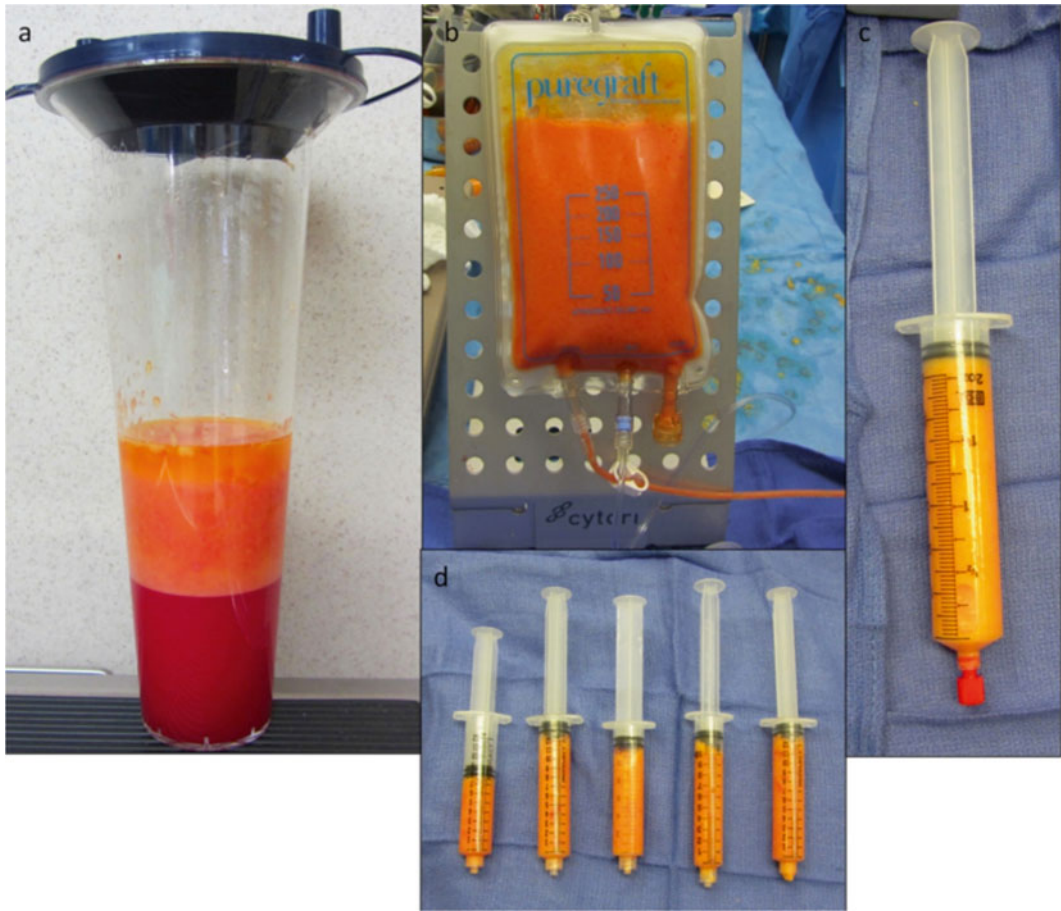


Fig. 1 Lipoaspirate obtained in the operating room. Lipoaspirate can be prepared for injection by (a) allowing it to separate by gravity and discarding the oily top layer and bottom supernatant or (b) by separating the fat with commercially available filtration; the PureGraft® system (Cytosol Therapeutics, San Diego, CA) is pictured here. The purified fat is re-collected with (c) 60 mL syringes and transferred to (d) 10 mL syringes for breast and body injection using blunt-tipped cannulas or 1 mL syringes for facial fat grafting

from the adipose matrix by adding Celase® reagent at an approximate concentration of 0.13 mg/cc of tissue processed. Alternatively, if the Celase® reagent is unavailable, a solution 0.075 % Collagenase I dissolved in DMEM is used to obtain 0.075 % Collagenase I (1 % penicillin/streptomycin) solution. The mixture can be sterilized and stored in liquid nitrogen at -20°C , if not needed for immediate use.

2. Two other solutions are prepared, a red cell lysis and a growth media solution. To prepare the former, solid NH_4Cl is dissolved in distilled water at 160 mM and vacuum sterilized with a $0.22\ \mu\text{m}$ filter prior to aliquoting. The growth media is prepared by adding 10 % FBS and 1 % penicillin/streptomycin solution to DMEM/F12 media and sterile filtering. Both of these solutions are stored at 4°C .

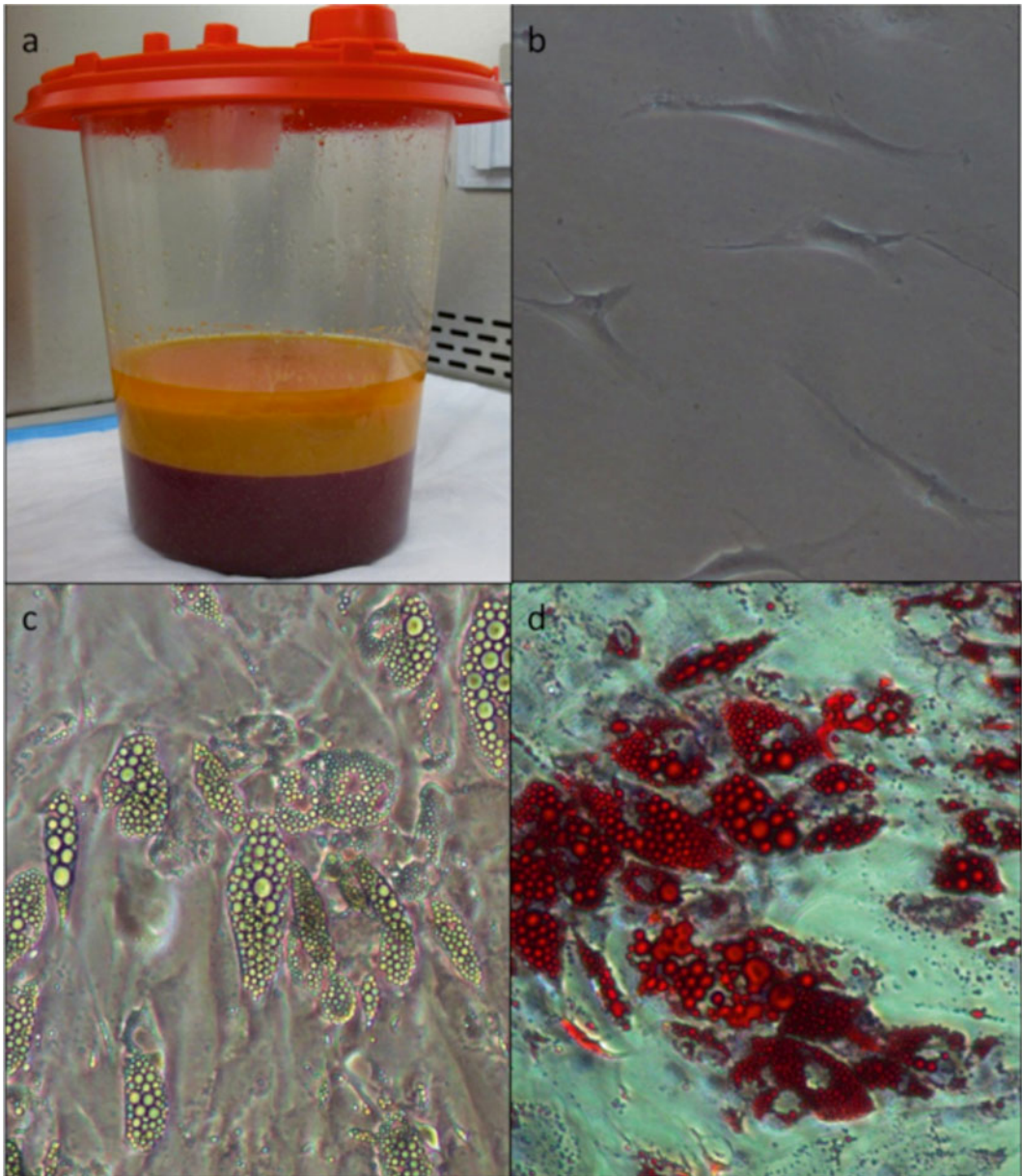


Fig. 2 Differentiation capabilities of naive ADSC. (a) Fat separation by gravity. (b) Naive adipose-derived stem cell under 40 $\times$ magnification. (c) Adipose-derived stem cells can be directed toward adipogenic differentiation in the laboratory, as evidenced by accumulating lipids within the intracellular vacuoles seen here under a bright-field microscope. (d) The Oil Red O dye stains neutral triglycerides and lipids bright red to confirm that these cells have indeed proceeded down an adipogenic lineage

3. Upon collection, the lipoaspirate sample will have separated into multiple layers (Fig. 2a). Attention is paid not to disturb the layers before collecting the adipose. The red, bottom layer is the tumescent fluid containing red blood cells, saline, lidocaine, and epinephrine. A clear yellow layer of free lipid oil forms

at the very top. The middle yellow-orange layer containing the fat tissue is then transferred into conical tubes containing the 0.075 % Collagenase I (1:1 v/v tissue:collagenase), as described above.

4. The conical tubes are then inverted several times to ensure that adipose tissue is fully exposed to the collagenase solution. The tubes are incubated at 37 °C for a total of 30 min on an orbital shaker with constant agitation.
5. After incubating an equal volume of growth media is added to each conical (total volume, approximately 12–14 mL) to neutralize the collagenase. The conicals are then centrifuged at 3,700 rpm for 10 min. The pellet at the bottom of the tube contains the SVF. The buoyant layer of adipocytes is carefully removed by aspiration without dislodging the SVF pellet.
6. Approximately 6 mL of supernatant is left in each tube and used to resuspend the SVF cells. Additional media is added to remove residual collagenase and then the cells are centrifuged again at 3,200 rpm for 2 min to reform the pellet.
7. The supernatant is discarded and, if the red blood cell lysis is desired, the cell pellet is resuspended in 2 mL of 160 mM NH_4Cl to lyse any remaining red blood cells. The tubes are then incubated at room temperature for 10 min.
8. Additional NH_4Cl can be added to lyse any remaining red blood cells. The admixture is centrifuged at 3,300 rpm for 10 min to re-pellet the ADSC-rich SVF fraction; the supernatant is aspirated and the pellet resuspended in 5 mL of growth media.
9. Next, the cell suspension is passed through a 70–100 μm cell strainer to eliminate major acellular debris and then rinsed with 5 mL additional growth media.

3.3 Culture of SVF

1. For culture, the suspension is plated into T-75 (75 cm^2) tissue culture flasks at a density of 100,000 cells/ cm^2 (approximately 1 flask for every ~6 mL of fat used). The flasks are labeled to indicate that this is the primary isolation passage ($P=0$) and incubated overnight at 37 °C in a humidified incubator with 5 % CO_2 .
2. After 24 h, the media from the flasks is aspirated and rinsed twice with warm PBS before fresh growth media is added. After 7–10 days the cells will reach 70–80 % confluency and should be passaged or frozen. They should not be allowed to reach 100 % confluency, as this can stimulate differentiation of the cells.

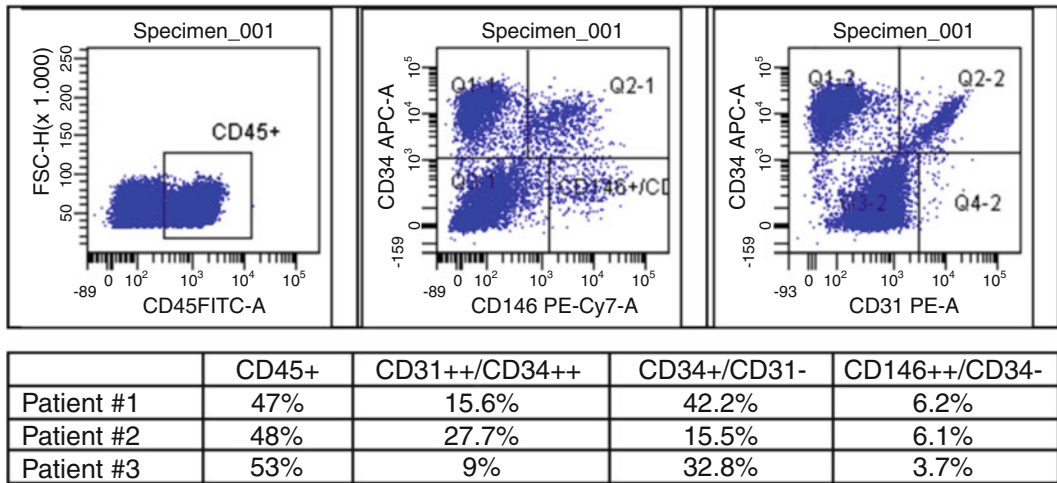


Fig. 3 Flow cytometric analysis of stromal vascular fraction. Assessment of cell surface marker expression from the SVF cells is accomplished by multiparametric flow cytometry. Cells are stained for the cell surface expression of CD31, CD34, CD45, and CD146. Although cells expressing these four markers were observed in all samples, the relative frequency of each subpopulation expressing any two of the markers used varied between patients. For instance, lipoaspirate from patient # 1 showed a higher number of leukocytes than the other two while lipoaspirate from patient # 2 showed a higher frequency of endothelial cells. Representative fluorescent dot plots using cells from a third patient are shown in this figure

3.4 Characterization of the SVF

3.4.1 Flow Cytometry

1. Cell characterization of SVF cells is routinely accomplished by using multiparametric flow cytometry for cell surface markers. In a recent study investigating the cell composition of small lipoaspirates from 30 patients undergoing fat grafting to the breast, we used a panel of four antibodies to phenotypically characterize SVF obtained from 50 mL fat samples donated by each patient. These included antibodies against human CD31, CD34, CD45, and CD146 (Fig. 3). CD146 is a member of the immunoglobulin superfamily and is expressed on endothelial cells, melanoma cells, and smooth muscle cells/pericytes. CD31, also known as platelet endothelial cell adhesion molecule-1 (PECAM-1), has wide tissue distribution and is expressed in high amounts on endothelial cells. CD45, also known as leukocyte common antigen (LCA), is a protein present on all human leukocytes. The CD34 antigen is a trans-membrane glycoprotein expressed on hematopoietic progenitor cells, vascular endothelium, bone marrow stromal cells, and embryonic fibroblasts. In adipose tissue, the CD34⁺ population harbors the majority of the ADSC population [64]. By analysis of the combined expression of these markers, it is possible to assess the frequency of leukocytes, endothelial cells, and smooth muscle-related and CD34⁺ progenitor cells in each sample.
2. For staining, the cell suspension is diluted in stain buffer (DPBS buffer supplemented with 2 % bovine serum albumin)

to achieve a concentration of 10^7 cells per mL. To reduce non-specific immunofluorescent staining, cells are incubated with murine antibody of the same isotype as test antibodies (1 mg/ 10^6 cells) for 15 min at 4 °C.

3. Appropriate fluorescence-minus-one (FMO) controls are used to mark the limits of the background staining. Then, approximately 5×10^5 cells are dispensed into each 12 × 75 mm polystyrene tube and primary antibodies are added (*see Note 7*). After incubation for 30 min at 4 °C in the dark, the cells are washed once in stain buffer and then fixed using FACS lysis buffer. Immunostained cells are assayed using FACSAria flow cytometer (BD Biosciences) and analyzed using FACSDiva software.
4. The crude SVF is a heterogeneous cell population including endothelial cells, pericytes, fibroblasts, leukocytes, and ADSCs. SVF cell yield and viability can be most accurately determined by using DNA-based methods. In the same study, by manually processing the adipose tissue using Celase® reagent, an average of 2.5×10^5 nucleated cells per gram of tissue with viability higher than 80 % was obtained (measures obtained using the NucleoCounter device (Chemometec, Denmark)). Under these conditions, an average of 26.7 % of SVF were found to have the phenotype CD34⁺/CD31⁻/CD45⁻, which is the fraction known to be enriched in ADSCs (Fig. 3).

3.4.2 CFU-F

1. The CFU-F (colony-forming unit fibroblast) assay is an in vitro technique used to quantify the frequency of adherent progenitor cells in a population (Fig. 4). For this, SVF cells are plated in low density (10^5 nucleated cells per cm²) and cultured for 12–14 days in a humid incubator with 5 % CO₂.
2. The media routinely used for culturing CFU-F from adipose is DME/F12 medium supplemented with 10 % fetal bovine serum and antibiotic/antimycotic solution (*see Note 8*).
3. The cultures are fed every 3–4 days and at the end of 14 days, the colonies are counted. This entails first washing the cells with PBS and then fixing the colonies by incubating the cultures in 10 % neutral buffered formalin.
4. After 15–20 min of incubation, the formalin is discarded and the cultures are rinsed twice with PBS. After the last wash, the cultures are stained with crystal violet solution (0.1 g crystal violet, 0.25 mL of glacial acetic acid, 100 mL of distill water) for 5–10 min at room temperature.
5. The crystal violet is then discarded, and the cultures washed gently with tap water and allowed to air-dry. The number of colonies (50 or more cells) and clusters (less than 50 cells) are then counted using an inverted microscope or stereoscope. In the study mentioned earlier, where small amounts of lipoaspirate were processed manually using the Celase® reagent, CFU-F was found to account for 1–1.5 % of SVF nucleated cells (*see Note 9*).

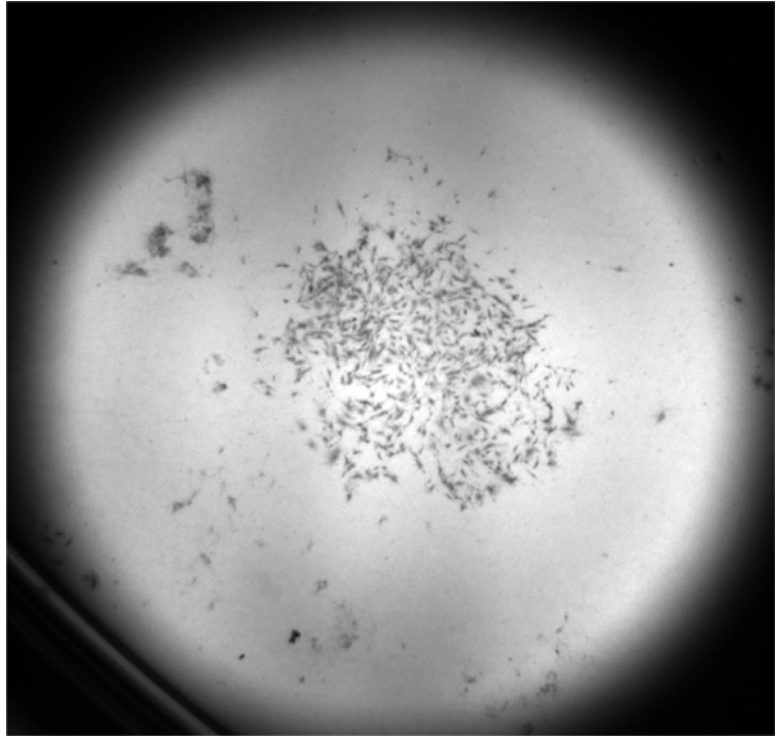


Fig. 4 Viable colony-forming units are capable of differentiation. The colony-forming unit fibroblast (CFU-F) in vitro assay to quantify the frequency of adherent progenitor cells in a population. For this, SVF cells are plated in low density (10^5 nucleated cells per cm^2) and cultured for 12–14 days in a humid incubator with 5 % CO_2 . The cultures are fed every 3–4 days and at the end of 14 days, the colonies are counted. The number of colonies (50 or more cells) and clusters (less than 50 cells) are then counted using an inverted microscope or stereoscope. In our studies, the CFU-F was found to account for 1–1.5 % of SVF nucleated cells

4 Notes

1. Preoperatively developing an order to the operative steps helps avoid wasted time while under anesthesia. For example, many practitioners will tumesce prior to setting up the OR field. This allows the epinephrine a chance to cause vasoconstriction without having “dead time.”
2. Consistent OR staff familiar with liposuction techniques and equipment greatly reduces wasted time and allows the surgeon to concentrate on the operation.
3. The adipocyte digestion process takes 90 min to complete when using the commercially available automated systems. Fat should be harvested at the beginning of the procedure if cells are to be re-injected as a mesotherapeutic agent or recombined with a scaffold, so that the patient is not under anesthesia unnecessarily long.

4. A super-wet or a tumescent infiltration technique can help avoid obtaining a bloody lipoaspirate. Further measures include allowing the tumescent to settle into the tissues for at least 15 min prior to aspiration.
5. The lipoaspirate will separate into three layers. Be careful not to shake the container and remix these layers.
6. If inconsistent fat grafting results are obtained, a bilaminar filtration system (e.g., PureGraft) can standardize the fat for regrafting and signal how much over-grafting may be necessary.
7. During the 10-min breaks, while the conical tubes are incubated at 37 °C for a total of 30 min, wipe the hood down the 70 % ethanol to sterilize the hood.
8. Leave the same amount of supernatant in each conical when aspirating the fat globule and discarding the aqueous layer (~6 mL).
9. Always remember to dispose of fat globs as biohazardous waste.

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Chapter 14

In Vitro Detection of Residual Undifferentiated Cells in Retinal Pigment Epithelial Cells Derived from Human Induced Pluripotent Stem Cells

Takuya Kuroda, Satoshi Yasuda, and Yoji Sato

Abstract

Human pluripotent stem cells (hPSCs) such as human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) are a leading candidate for regenerative medicine/cell therapies because of their capacity for pluripotency and unlimited self-renewal. However, there are significant obstacles preventing the clinical use of hPSCs. A significant safety issue is the presence of residual undifferentiated cells that have the potential to form tumors in vivo. Here, we describe the highly sensitive qRT-PCR methods for detection of residual undifferentiated cells in retinal pigment epithelial (RPE) cells derived from hiPSCs. qRT-PCR using probes and primers targeting *LIN28A* (*LIN28*) transcripts can detect residual undifferentiated cell levels as low as 0.002 % in hiPSC-derived RPE cells. We expect this method to contribute to process validation and quality control of hiPSC-derived cell therapy product.

Key words Human induced pluripotent stem cells, Retinal pigment epithelial cells, Tumorigenicity, *LIN28*, qRT-PCR

1 Introduction

Pluripotent stem cells such as human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) are pluripotent and have the ability to self-renew. Pluripotency is the ability to differentiate into a variety of cells and self-renewal is the ability to undergo numerous cell division cycles while maintaining cellular identity. Because of these two characteristics, it has been expected that these cell types will provide new sources for robust and continuous production of a variety of cells and tissues for regenerative medicine/cell therapy. Additionally, hiPSCs provide a possible solution to the ethical problems and the immune rejection of hESC-derived cells, thus raising novel avenues for patient-specific cell therapy. As previously reported [1, 2], many attempts are currently under way to differentiate hESCs and hiPSCs into various tissues. Cell therapy using hESCs or iPSCs has already entered

the scope of clinical application. Indeed, a clinical trial using hESC-derived-RPE cells for Stargardt's disease and the dry type of age-related macular degeneration (dry AMD) have been initiated [3]. Clinical research using autologous hiPSC-derived RPE for the wet type of age-related macular degeneration (wet AMD) is also being planned in Japan.

One of the most important issues in the development of a safe pharmaceutical or medical device derived from hPSCs is ensuring that the final product does not form tumors after implantation [4]. There are two primary concerns. First, the pluripotent stem cell-based product may be unstable and transform to produce a tumor under some specific environment though the genomic stability of differentiated cells derived from hESCs/hiPSCs is currently unclear. Second, the product derived from human pluripotent stem cells might contain residual undifferentiated stem cells that would eventually proliferate and form a teratoma [5]. To address this second concern, it is critical to develop highly sensitive assays for detection of residual undifferentiated stem cells in the final products, and to determine their lower limit of detection (LLOD). An evaluation study of the in vivo tumorigenicity assay using severe combined immunodeficiency (SCID) mice has shown that 245 undifferentiated hESCs spiked into 10^6 feeder fibroblasts produce a teratoma [6]. However, some in vitro assays, such as quantitative real-time polymerase chain reaction (qRT-PCR), flow cytometry, and immunohistochemistry, have been used to indicate the undifferentiated state of stem cells with various markers (such as *POU5F1* (*OCT3/4*), *NANOG*, *SOX2*, *LIN28*, *MYC* (*C-MYC*), *REX1*, *KLF4*, *TRA-1-60*, *TRA-1-81*, *SSEA-3*, and *SSEA-4*) [7, 8]. We have demonstrated that the qRT-PCR assay can successfully detect as low as 0.002 % residual undifferentiated hiPSCs in hiPSC-induced RPE cells using *LIN28* as a target gene (designated as the *LIN28*/qRT-PCR method) [9]. To the best of our knowledge, the *LIN28*/qRT-PCR method is the most sensitive of the previously reported other methods in detecting undifferentiated PSCs. In this chapter, the *LIN28*/qRT-PCR protocol used in our laboratories is described in detail.

2 Materials

1. Pipetman (2, 10, 20, 200, and 1,000 μ L).
2. Incubator, humidified, 37 °C, 5 % CO₂.
3. Sterile biosafety cabinet.
4. Liquid disposal system for aspiration.
5. Centrifuge.
6. Sampling tubes (1.5 mL).

7. Sterile serological pipets (5, 10, and 25 mL).
8. Sterile conical tubes (15 and 50 mL).
9. Cell culture plates (60 and 100 mm).
10. Tips (10, 20, 200, and 1,000 μ L).

2.1 Maintenance and Culture of hiPS, RPE Cell, and SNL Cell

1. The hiPSC line 201B7 induced by transducing *OCT3/4*, *SOX2*, *KLF4*, and *C-MYC* (RIKEN Cell Bank).
2. Normal human retinal pigment epithelial cells (Lonza Biologics, 00194987).
3. SNL cells (a mouse fibroblast STO cell line expressing the neomycin-resistance gene cassette and LIF, CELL BIOLABS, CBA-316).

2.2 Cell Culture

2.2.1 SNL Culture

1. Dulbecco's modified Eagle medium (DMEM).
2. Phosphate-buffered saline (PBS), calcium free, magnesium free.
3. Fetal bovine serum (FBS).
4. L-Glutamine.
5. Penicillin/streptomycin.
6. Gelatin: To make 0.1 % gelatin solution, dissolve 1.0 g of gelatin powder in 1,000 mL of distilled water, autoclave, and store at 4 °C.
7. SNL medium, 500 mL: Mix 500 mL DMEM, 38.5 mL FBS, 5.5 mL L-glutamine, and 3 mL penicillin/streptomycin.
8. 0.25 % Trypsin/EDTA solution.
9. Mitomycin C.

2.2.2 hiPSC Culture

1. Primate ES Cell medium (ReproCELL, RCHEMD001).
2. Penicillin/streptomycin.
3. Recombinant human FGF basic 146 aa (bFGF).
4. hiPSC medium, 500 mL: Mix 500 mL primate ES cell medium and 2.5 mL penicillin/streptomycin. Add 4 ng/mL bFGF into the medium before use. Store at 4 °C for up to 2 weeks.
5. PBS (*see* Subheading [2.2.1](#)).
6. CTK solution (ReproCELL, RCHETP002).
7. StemPro EZPassage.
8. Cell scraper.
9. 100 μ m cell strainer.

2.2.3 Primary PRE Cell Culture

1. RtEBM Bullet Kit (RtEBM medium; Lonza).
2. PBS (*see* Subheading [2.2.1](#)).
3. 0.25 % Trypsin/EDTA solution.
4. FBS (contained in RtEBM Bullet Kit).

2.3 Total RNA Extraction

1. RNeasy Mini Kit.
2. QIAshredder.
3. RNase-free DNase set.
4. Cell scraper.

2.4 qRT-PCR

1. QuantiTect Probe one-step RT-PCR Kit.
2. 96-Well PCR plate.
3. Dual-labeled probe and primer.
4. TaqMan GAPDH control reagents, human.
5. Applied Biosystems 7300 Real Time PCR systems.

3 Methods

3.1 Cell Culture

3.1.1 MMC-Treated SNL Cells

1. Add 3 or 5 mL of gelatin solution into a 60 or a 100 mm dish and incubate for 30 min.
2. Aspirate gelatin solution and use this dish as gelatin-coated dish.
3. Thaw quickly the 1×10^6 cells of cryopreserved SNL cells in water bath at 37 °C and suspend with 9 mL of SNL medium in a 15 mL tube.
4. Centrifuge the 15 mL tube at 170×g for 5 min and aspirate supernatant.
5. Suspend SNL cell pellet with 10 mL of SNL medium and transfer into a gelatin-coated 10 cm dish.
6. Culture in a humidified atmosphere of 95 % air with 5 % CO₂ at 37 °C (*see Note 1*).
7. When SNL cells reach confluence, add 12 µg/mL MMC and incubate for 2 h and 30 min.
8. Wash the MMC-treated SNL cells twice with 10 mL of PBS.
9. Detach the cells with 1 mL of 0.25 % trypsin/EDTA solution, add 10 mL of SNL medium, and then transfer into 15 mL tube.
10. Centrifuge the 15 mL tube at 170 g for 5 min and aspirate supernatant.
11. Suspend the cell pellet with 10 mL SNL medium, transfer the cell suspension to a 60 mm dish (5×10^5 cells/dish), and incubate in a humidified atmosphere of 95 % air with 5 % CO₂ at 37 °C for 1 day.

3.1.2 hiPSC Culture on Feeder Cells

1. Culture hiPSC colonies in 60 mm dish (pre-seeded with MMC-SNL feeder cells) until colonies reach 70–80 % confluency. Aspirate the medium, and wash twice the cells with 5 mL of PBS per dish.

2. Remove PBS completely, add 0.5 mL CTK solution, and incubate at 37 °C for 3 min (*see Note 2*).
3. Aspirate CTK solution, and wash twice with 5 mL of PBS.
4. Remove PBS completely and add 5 mL of hiPSC medium.
5. Cut hiPSC colonies using EZPassage (*see Note 3*).
6. Detach hiPSC colonies using a cell scraper.
7. Resuspend the cell clump in 5 mL of hiPSC medium and through 100 µm cell strainer.
8. Transfer the cell clump suspension to a 15 mL tube. Centrifuge at 100×*g* for 5 min, and then aspirate the supernatant.
9. Resuspend the cell clump in 5 mL of hiPSC medium and transfer the cell clump suspension onto a 60 mm dish that is pre-seeded with MMC-SNL feeder cells.
10. Incubate at 37 °C and 5 % CO₂ until cells reach 70–80% confluency (*see Note 4*).

3.1.3 Primary RPE Cell Culture

1. Culture primary RPE cells in 100 mm dish for 14 days.
2. Aspirate the medium, and wash the cells with 10 mL PBS.
3. Remove PBS completely, add 0.5 mL 0.25 % trypsin/EDTA solution, and incubate at 37 °C for 10 min. Add 10 mL RtEBM medium (with 2 % FBS), and then transfer the cell suspension to a 15 mL tube.
4. Centrifuge at 200×*g* for 5 min, and then discard the supernatant.
5. Resuspend the cell in 5 mL of RtEBM medium (with 2 % FBS).
6. Seed 1 × 10⁶ cells into a 60 mm dish (*see Note 5*).

3.2 RPE Cell Differentiation of hiPSCs

The procedure for differentiating hiPSCs into RPE cells was performed as previously described [9, 10].

3.3 Total RNA Extraction

1. Extract total RNA using the RNeasy Mini Kit, according to the manufacturer's instructions (*see Note 6*).
2. The lysate was homogenized using a QIAshredder and on-column DNase digestion was performed according to the manufacturer's instructions.
3. The RNA quantity and quality were determined by measuring the absorbance at 260 and 280 nm using NanoDrop. The RNA concentration was calculated and the RNA samples were stored at –80 °C.

3.4 One-Step qRT-PCR

1. PCR primers and dual-labeled probes are listed in Table 1.
2. Preparation of a reaction mix using the QuantiTect Probe RT-PCR kit. The primer and probe concentrations were

Table 1
qRT-PCR probes and primers [9]

Gene	Probe sequences (5' → 3')	Forward primer sequences (5' → 3')	Reverse primer sequences (5' → 3')
<i>NANOG</i>	TGCTGAGGCCCTTCTGCGTCACACC	CTCAGCTACAAACAGGTGAAGAC	TCCCTGGTGGTAGGAAGAGTAAA
<i>OCT3/4</i>	CGGACCACATCCTTCTCGAGCCCAAGC	GAAACCCACACTGCAGCAGA	TCGCTTGCCCTTCTGGCG
<i>LIN28</i>	CGCATGGGGTTCGGCTTCCCTGTCC	CACGGTGCGGGGCATCTG	CCTTCCATGTGCAGCTTACTC
<i>REX1</i>	AGCAAAACACCTGCTGGACTGTGAGCAC	CCATCGCTGAGCTGAAACAAA	CCTCCAGGCAGTAGTGATCTG
<i>C-MYC</i>	TTGTTCCCTCCTCAGAGTCGCTGCTGGT	GCTCCATGAGGAGACACCG	CCACAGAAAACACATCGATTCTTC
<i>SOX2</i>	CTCGCAGACCTACATGAACGGCTCGC	GCGCCCTGCAGTACAACCTC	CGGACTTGACCAACCGAACC
<i>KLF4</i>	ACCTGCGAAACCCACACAGGTGAGAAAC	CCTACACAAAGAGTTCCCATCTCA	CCGTCCCAGTCACAGTGGTA

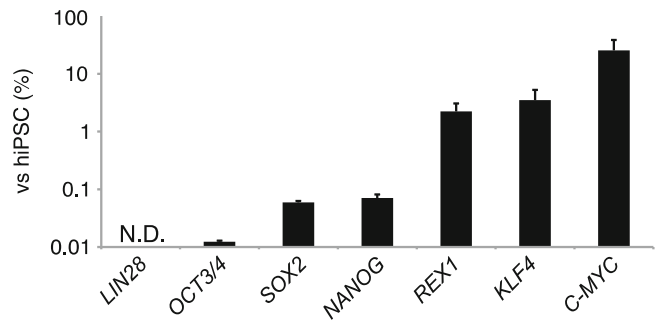


Fig. 1 The relative mRNA expressions in primary RPE cells of *LIN28*, *OCT3/4*, *SOX2*, *NANOG*, *REX1*, *KLF4*, and *C-MYC* were determined by qRT-PCR analysis [9]. All values are expressed as mRNA levels relative to those in undifferentiated hiPSCs. Results are means ± standard deviation (n=3)

determined to be 400 nM and 100 nM, respectively, in a final reaction volume of 25 µl. 250 ng of total RNA (5 µL of 50 ng/µL total RNA) was used as template (see Note 7). Prior to amplification, the contents of each reaction were mixed by spinning the plate for 2 min at 700×g. The optimal thermal cycling conditions were 50 °C for 30 min and 95 °C for 15 min followed by 45 cycles of 94 °C for 15 s and 60 °C for 1 min.

3.5 Quantification

Data analysis based on a standard curve method: A 201B7 total RNA dilution series (10, 3, 1, 0.3, 0.1, 0.03, 0.01, 0.003, and 0.001 ng/µL) was prepared as a standard curve.

Target gene expression levels were normalized to those of the GAPDH transcript, which were quantified using TaqMan human GAPDH control reagents.

3.6 Detection of Undifferentiated hiPSCs Using qRT-PCR

3.6.1 Identification of Highly Selective Markers for Undifferentiated hiPSCs

To identify highly selective markers for undifferentiated hiPSCs, we compared the *OCT3/4*, *KLF4*, *C-MYC*, *SOX2*, *NANOG*, *LIN28*, and *REX1* mRNA levels in hiPSCs and primary RPE cells (Fig. 1). Primary RPE cells were found to endogenously express *C-MYC* at 25.49 % of the levels observed in hiPSC, which was consistent with the previous finding that MEF expressed *C-MYC* at approximately 20 % of levels observed in mouse ES cells [11]. *KLF4* and *REX1* expression levels in primary RPE cells were 3.51 % and 2.23 %, respectively, compared with hiPSCs. However, there was more than a 1,000-fold difference in *NANOG* (0.07 %), *SOX2* (0.06 %), *OCT3/4* (0.01 %), and *LIN28* (not detected) gene expression between primary RPE cells and hiPSCs. These results suggested that the latter four genes are useful in detecting hiPSCs in RPE cells.

3.6.2 hiPSC-Spiked Sample Analyzed Using the Lin28/qRT-PCR Method

We then conducted two sets of qRT-PCR assays to evaluate the utility of these marker genes to detect spiked hiPSCs in primary RPE cells. We first measured *NANOG*, *OCT3/4*, and *LIN28* mRNA levels in five lots of primary RPE cells (the negative control)

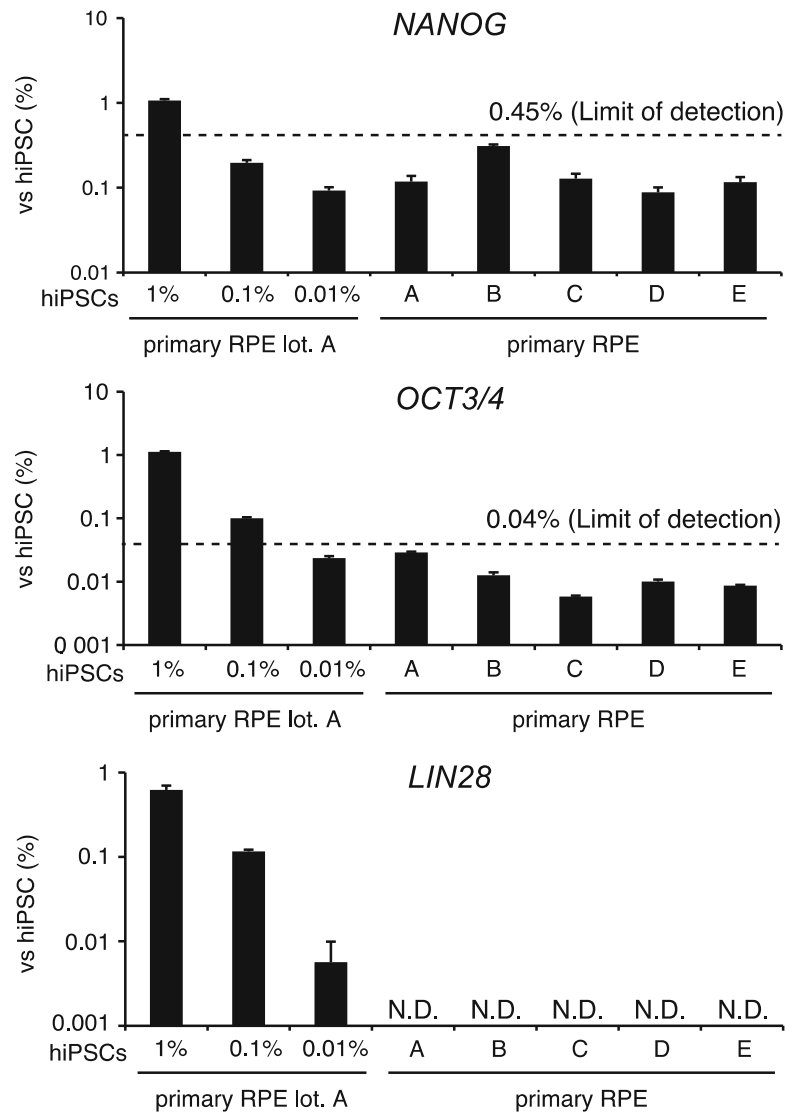


Fig. 2 qRT-PCR analysis of hiPSCs spiked into primary RPE cells and five lots of primary RPE cells [9]. Single-cell hiPSCs were spiked into primary RPE cells, and total RNA was isolated from the mixed cells. The mRNA levels of *NANOG*, *OCT3/4*, and *LIN28* are shown as a relative expression. Limit of detection was calculated as the mean plus 3.3-fold the standard deviation of the measurement of the five lots of primary RPE cells. All values are expressed as mRNA levels relative to those in undifferentiated hiPSCs. Results are means $\pm$ standard deviation ($n=3$)

to determine the LLOD. Limit of detection was calculated as the mean plus 3.3-fold the standard deviation of the measurement of the five lots of primary RPE cells [12]. The LLOD for *NANOG* and *OCT3/4* mRNA was 0.45 % and 0.04 %, respectively, compared with hiPSCs (Fig. 2). The LLOD for *LIN28* could not be calculated because no fluorescent signal for *LIN28* expression was detectable in the primary RPE cells. These results along with

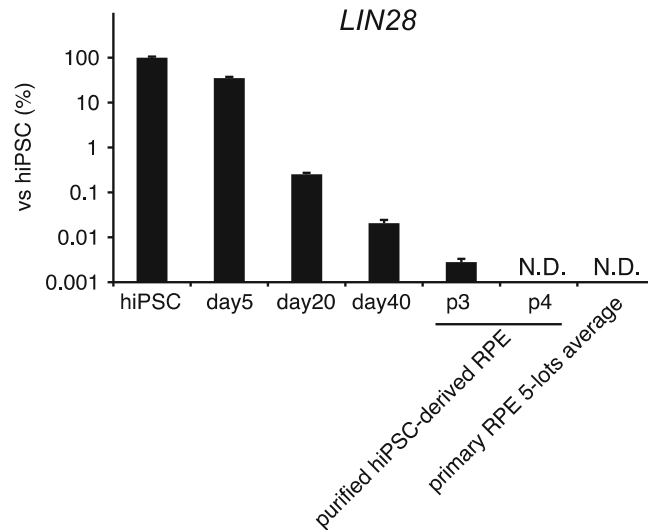


Fig. 3 *LIN28* expression of hiPSCs differentiating into RPE and purified hiPSC-derived RPE cells (passages 3 and 4) [9]. All values are expressed as mRNA levels relative to those in undifferentiated hiPSCs. Results are means $\pm$ standard deviation ($n=3$)

experiments spiking 2.5×10^4 (1 %), 2.5×10^3 (0.1 %), and 2.5×10^2 (0.01 %) hiPSCs into 2.5×10^6 primary RPE cells (Fig. 2) indicated that measuring *NANOG*, *OCT3/4*, and *LIN28* expression using qRT-PCRs detected at least 1 %, 0.1 %, and 0.01 % contamination of residual undifferentiated hiPSCs in RPE cells, respectively.

3.6.3 hiPSC-Derived RPE Analyzed Using the *LIN28*/qRT-PCR Method

We examined whether qRT-PCR analysis of *LIN28* was able to detect residual undifferentiated cells in differentiated RPE cells from hiPSCs. Total RNA extracted from the differentiating cells (days 5, 20, and 40) and purified hiPSC-derived RPE cells (passages 3 and 4) underwent qRT-PCR analysis. The *LIN28* mRNA level was continuously downregulated during the differentiation process, being 0.02 % that of hiPSCs at day 40. In early passage culture (passage 3) after purification, *LIN28* was still significantly expressed at a level 0.002 % that of hiPSCs. From passage 4 onward, however, there was no detectable level of *LIN28* expression observed in hiPSC-derived RPE cells (Fig. 3). These results suggest that the *LIN28*/qRT-PCR method detects 0.002 % of residual undifferentiated hiPSCs in hiPSC-induced RPE cells, which indicates that a single hiPSC in 5.0×10^4 RPE cells is detectable.

4 Notes

1. Change the medium every 3 days until the next passage step.
2. To prevent inactivation of CTK, do not warm 37 °C before use. Make sure that feeder cells are dissociated, while hiPS colonies

- remain attached on the plate. Then, remove CTK solution as soon as possible to maintain good viability of the harvested cell.
3. Cuts hiPS colonies into uniform-sized pieces for reproducible and optimal passaging.
4. Change the medium every day until the next passage step.
5. Next day, make sure that RPE cells are attached on plate, and change the medium with RtEBM medium (without FBS). Change the medium every 2–3 days until the next passage step.
6. In the spiking study, 201B7 and RPE cells were mixed at a defined cell number, before total RNA extraction.
7. If sufficient total RNA sample were not available, it is possible to use 50 ng total RNA as qRT-PCR template.

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Whole-Mount Immunohistochemistry to Study Spermatogonial Stem Cells and Spermatogenic Lineage Development in Mice, Monkeys, and Humans

Kathrin Gassei, Hanna Valli, and Kyle E. Orwig

Abstract

Spermatogonial stem cells (SSCs) and undifferentiated progenitor spermatogonia in mammalian seminiferous tubules are organized in chains, connected by intracellular bridges. Clone size is generally related to stem cell potential, with shorter chains containing the majority of the stem cell population. Immunofluorescence detection of spermatogonia-specific proteins in whole-mount seminiferous tubule preparations is the only method that allows researchers to relate clone size with the molecular phenotype in spermatogenic lineage development. Here we describe in detail the method used to detect nuclear, cytoplasmic, and cell surface molecules in seminiferous tubules isolated from mouse, monkey, and human testes.

Key words Seminiferous tubule, Spermatogonia, Whole mount, Molecular marker, Clonal development, Germ lineage, Immunofluorescence microscopy

1 Introduction

Spermatogenesis is driven by spermatogonial stem cells (SSCs) that are located at the basement membrane of the seminiferous tubules. SSCs balance self-renewal and differentiation in response to external signals to maintain the stem cell pool and to produce large numbers of mature sperm over a long period of time. SSC self-renewal leads to the formation of daughter cells with stem cell activity to maintain the stem cell pool. A differentiation fate decision produces diploid progenitor cells termed undifferentiated spermatogonia, which are characterized by their heterochromatin-free nuclear morphology and have been termed type A spermatogonia [1]. In mouse, daughter cells from transit-amplifying divisions remain connected by cytoplasmic bridges and form chains of 2, 4, 8, 16, or more spermatogonia (A aligned or A_{al} spermatogonia) [2, 3]. Based on their morphology, SSCs are indistinguishable from undifferentiated progenitor spermatogonia, but they can be identified

through germ cell transplantation and repopulation of seminiferous tubules of infertile recipient animals [4]. Germ cell transplantation is the gold standard assay to determine stem cell activity, but by its nature it is a retrospective assay and does not allow the researcher to prospectively identify SSCs. It is widely accepted that SSC activity resides, at least in part, in the pool of type A single (A_s) spermatogonia [5] and more recently it has been suggested that stem cell activity may extend to some pairs and chains of four spermatogonia [6, 7, 8].

In nonhuman primates and humans, the stem cells are the A_{dark} and A_{pale} spermatogonia, characterized by their hematoxylin staining intensity. The A_{dark} spermatogonia are considered to be the quiescent or reserve stem cells, while the A_{pale} spermatogonia are the active stem cells. The A_{pale} spermatogonia will either self-renew (perhaps reverting to an A_{dark} phenotype) or go on to produce B spermatogonia which will go through one (human) to four (non-human primate) mitotic divisions before initiating meiosis and morphological differentiation to produce sperm [9–14].

Our current understanding of the clonal organization of stem and progenitor spermatogonia in rodents is derived from extensive histological studies on whole-mounted seminiferous tubules by Huckins and Oakberg [3, 15, 16]. These studies are the basis of the A_s model of SSC self-renewal in rodents as described above. For the method of whole-mount immunohistochemistry, intact seminiferous tubules are dissected, fixed, and stained with histological stains. This method offers great advantages over using testicular tissue cross sections for spermatogonial evaluation because it is possible to describe the three-dimensional arrangement of spermatogonia and to determine clone sizes that may be related to stem cell potential. The different types of spermatogonia can be identified by comparing nuclear morphology, nuclear-to-cytoplasm ratio, location within the tubules, and chain length. Thus, we now have a detailed understanding of the progression of spermatogenesis for many different species, including monkeys and humans [9, 17, 18].

However, the molecular mechanisms that regulate self-renewal and differentiation fate decisions are still widely unknown. One of the most significant technical advances in recent years to study spermatogonia has been the combination of whole-mount seminiferous preparations with immunofluorescence staining of proteins using specific antibodies. This method added another level of detail to our understanding of spermatogenic lineage development that could not be achieved in testicular tissue cross sections alone. A number of transcription factors (SALL4 [19], PLZF [20], ID4 [21], UTF1 [22], SOHLH1 [23] and SOHLH2 [24]), cytoplasmic proteins (LIN28 [25], NANOS2 and NANOS3 [26]), and cell surface molecules (GFR α 1 [27], GPR125 [28], CDH1 [29]) with an expression pattern restricted to undifferentiated spermatogonia has been described, and antibodies directed against these molecules can be used to identify undifferentiated spermatogonia

Table 1
Commercially available antibodies for testis whole-mount staining

	Antigen	Antibody	Protocol	Dilution
Mouse	SALL4	Mouse (Abcam, ab57577)	3 days or overnight	1:200–1:400
		Rabbit (Abcam, ab29112)	3 days or overnight	1:800–1:1,000
	PLZF	Goat (R&D systems, AF2944)	3 days or overnight	1:50–1:200
	LIN28	Goat (R&D systems, AF3757)	3 days or overnight	1:200
	GFRa1	Goat (R&D systems, AF560)	Overnight	1:10
	CDH1	Goat (R&D systems, AF748)	Overnight	1:100–1:200
	UTF1	Rabbit (Abcam, ab24273)	Overnight	1:100
nhp/ human	SALL4	Rabbit (Abcam, ab29112)	3 days or overnight	1:200–1:500
	PLZF	Goat (R&D systems, AF2944)	Overnight	1:50
	UTF1	Mouse (Millipore, MAB4337)	Overnight	1:500
	PGP9.5	Rabbit (Biogenesis, 7863-0507)	Overnight	1:100–1:500
	TEX14	Rabbit (Proteintech, 18351-1-AP)	Overnight	1:50
	cKIT	Goat (R&D, systems AF332)	Overnight	1:50

and potential SSCs. Using more than one marker in co-staining experiments further revealed phenotypic heterogeneity among otherwise similar spermatogonia, which might indicate functional heterogeneity [19, 25, 30].

In this chapter, we provide detailed protocols for the immunodetection of nuclear, cytoplasmic, and cell surface marker proteins in rodent, monkey, and human whole-mount seminiferous tubules (Table 1).

2 Materials

2.1 Requirements

1. 15 mL tubes to mix enzyme solutions.
2. 60 mm dishes for tissue dissection and tubule digestion.
3. Dissecting microscope.
4. Dissecting tools: Scissors and very fine tweezers.
5. Glass scintillation vials for tissue fixation.
6. 12-Well cell culture plate with 15 mm net-well insert (74 μ m mesh size) and 15 mm Corning Netwell Carrier Kit #3520 for the washing steps of the staining protocol (Fig. 1).



Fig. 1 Net wells used to incubate samples

7. 48-Well culture plates for antibody incubation: For larger samples it might be necessary to use 24-well plates and a larger incubation volume to allow for sufficient antibody diffusion. If only a very small number of tubules are stained, or rare antibodies are used, incubation can be in 96-well plates and in a reduced volume.
8. Shaker incubator for antibody incubation.
9. Microscopy slides, glass cover slips (50×22 mm), and nail polish for mounting and sealing of seminiferous tubules.

2.2 Buffers and Staining Solutions

1. Enzyme solution for tubule digestion: Dissolve 1 mg/mL DNase I and 1 mg/mL Collagenase IV in HBSS. Prepare 3 mL for each pair of testes to be digested (mouse, scale up accordingly based on tissue volume). Prepare fresh right before seminiferous tubule preparation.
2. Fixative: Prepare 4 % paraformaldehyde in Dulbecco's phosphate-buffered saline (DPBS) and adjust pH to 7.2. Prepare fresh every week and store at 4 °C.
3. Blocking buffer (PBSMT): Prepare sufficient volumes of 2 % Biotto dry milk + 0.5 % Triton X-100 in DPBS and store at 4 °C.
4. Dehydration: Prepare sufficient volumes of 25, 50, 75, and 95 % methanol in DPBS. Prepare fresh for every experiment.

5. Permeabilization and fixation: Prepare volumes of a methanol:DMSO:H₂O₂ mixture at a 4:1:1 ratio. Prepare fresh for every experiment.
6. Washing buffer (PBSTx): 0.1 % Triton X-100 in DPBS. Sterile filter and store at room temperature.

3 Methods

3.1 Preparation of Whole-Mount Seminiferous Tubules (Mouse)

1. Mice are sacrificed and testes recovered for analysis.
2. Remove tunica, gently tease seminiferous tubules apart with fine forceps, and digest with 1 mg/mL DNase I and 1 mg/mL Collagenase IV in HBSS (*see Note 1*).
3. Seminiferous tubules are fixed for a minimum of 2 h in 4 % PFA at 4 °C (*see Note 2*).
4. Wash tubules extensively in PBS 3–4× at 60-min intervals.
5. *Possible stop point*: Seminiferous tubules can be stored in PBS at 4 °C (*see Note 3*).

3.2 Preparation of Whole-Mount Seminiferous Tubules (Nonhuman Primate and Human)

1. Remove tunica and cut a piece of nonhuman primate or human testis.
2. Gently tease seminiferous tubules apart with fine forceps, and digest with 1 mg/mL DNase I and 1 mg/mL Collagenase IV in PBS (*see Note 1*).
3. Seminiferous tubules are fixed overnight in 4 % PFA at 4 °C (*see Note 2*).
4. Wash tubules extensively in PBS 3–4× at 60-min intervals.
5. *Possible stop point*: Seminiferous tubules can be stored in PBS at 4 °C (*see Note 3*).

3.3 Whole-Mount Immunofluorescence Detection of Nuclear Proteins

Examples of the expected staining patterns are shown in Fig. 2.

Dehydration, fixation, rehydration, blocking, and primary antibody incubation (day 1) (see Note 4).

1. For most incubations, samples are processed in net-well tissue culture inserts (12-well, 12 µm pore). Washes are removed by aspirating from bottom chamber (Fig. 1).
2. Transfer testes into 12-well net-well plates with 3 mL PBS per well. Sequentially wash in 3 mL MeOH/DPBS (25, 50, 75, 95 %, and 2× in 100 % MeOH) for 10 min on rocker at room temperature.
3. *Possible stop point*: Samples can be stored in 100 % MeOH at –20 °C for up to 1 month.
4. Incubate samples in 3 mL fresh MeOH:DMSO:H₂O₂ (4:1:1) at room temperature for 3 h.

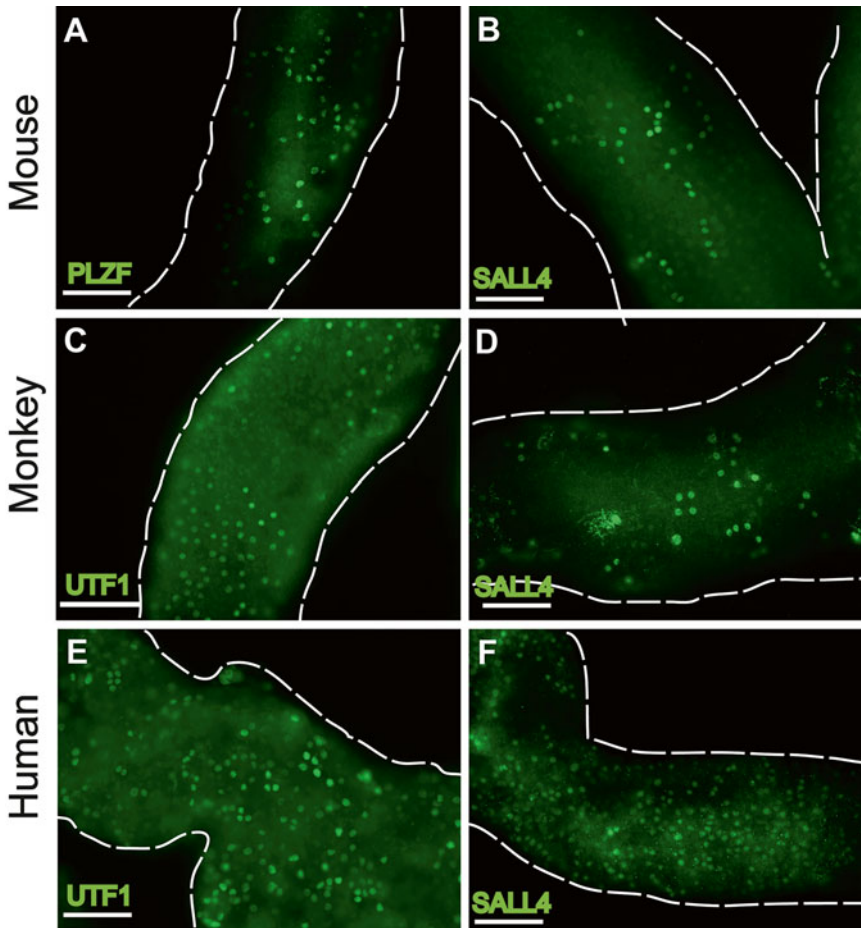


Fig. 2 Whole-mount staining for nuclear markers in mouse, monkey, and human testis. Staining for nuclear markers (a) PLZF and (b) SALL4 in mouse testis; (c) UTF1 and (d) SALL4 in monkey testis, and (e) and (f) in human testis. Scale bar = 100 μ m

5. *Possible stop point:* Samples can be stored in 100 % MeOH at -20°C for up to 1 month.
6. Sequentially rehydrate samples in 3 mL 50 % MeOH/DPBS (10 min), 25 % MeOH/DPBS (10 min), and PBS (2×15 min).
7. Block samples with 3 mL fresh ice-cold PBSMT (2×15 min, $1 \times 1-2$ h).
8. Transfer tubules to 24/48/96-well plate (according to sample size) and incubate in an appropriate volume (300, 200, 100 μ l, respectively) of primary antibody solution in PBMST overnight at 4°C .

Secondary antibody incubation (day 2)

9. Transfer samples back to net-well plate.
10. Wash with freshly prepared ice-cold 3 mL PBSMT (2×15 min, 5×1 h).

11. Transfer back to 24/48/96-well plate and incubate with fluorescent dye-conjugated secondary antibody in PBSMT overnight at 4 °C (e.g., FITC, TRITC; same volumes as above) (*see Note 5*).
12. Cover from light during all subsequent steps to prevent photobleaching of fluorescent dye.
13. Transfer sample back to net-well plate and wash with freshly prepared ice-cold 3 mL PBSMT (2 × 15 min, 5 × 1 h).
14. Wash with 3 mL DPBS 2 × 10 min at room temperature and proceed with Subheading 3.5 to mount tubules (see below).

3.4 Whole-Mount Immunofluorescence Staining of Cytoplasmic and Surface Membrane Proteins

Examples of the expected staining patterns are shown in Fig. 3.

Dehydration, fixation, rehydration, blocking, and primary antibody incubation

1. Transfer tubules to net-well tissue culture inserts and wash with PBSTx for 15 min at room temperature.
2. Incubate tubules in 10 % methanol in PBSTx for 1 h at 4 °C.
3. Block unspecific binding sites with blocking buffer PBSMT for 1 h at room temperature.
4. Dilute antibody in PBSMT and incubate at 37 °C for a minimum of 60 min in a 24/48/96-well plate (same volumes as above).

Secondary antibody incubation

5. Transfer samples back to net-well plate.
6. Wash with freshly prepared ice-cold 3 mL PBSMT 6 × 15 min.
7. Transfer back to 24/48/96-well plate and incubate with fluorescent dye-conjugated secondary antibody in PBSMT at 37 °C for a minimum of 60 min in a 24/48/96 (same volumes as above) (*see Note 5*). Cover from light during all subsequent steps to prevent photobleaching of fluorescent dye.
8. Transfer samples back to net-well plate
9. Wash with freshly prepared ice-cold 3 mL PBSMT 6 × 15 min.
10. Wash with 3 mL DPBS 2 × 10 min at room temperature.

3.5 Mounting and Imaging of Whole-Mount Seminiferous Tubules

1. Prepare raised-coverslip glass slides by placing four small drops of nail polish in each corner of a conventional microscopy slide. Let the nail polish dry for 15 min. Slides can be prepared ahead of time (Fig. 4).
2. Remove samples from net-well plate and transfer to raised-coverslip slides. Gently spread tubules using a dissecting scope and fine tweezers.
3. Mount cover slips perched on nail polish drops with 1–2 drops of Vectashield w/DAPI mounting medium (*see Note 6*).

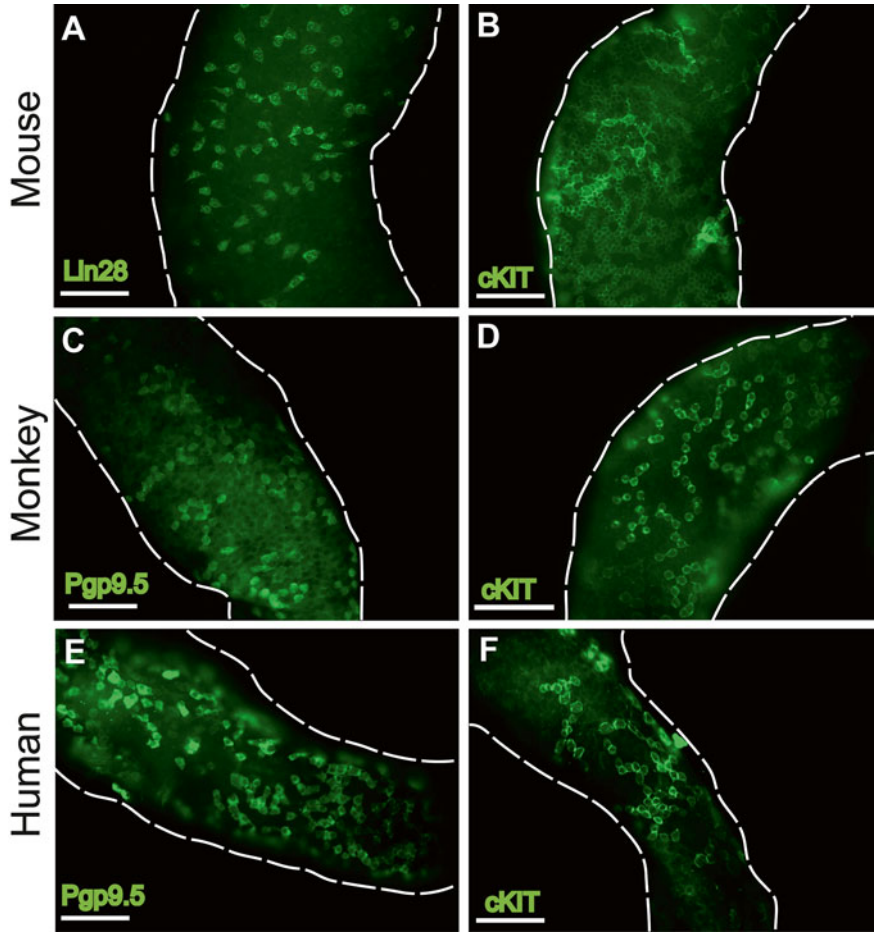


Fig. 3 Whole-mount staining for cytoplasmic and cell-surface markers in mouse, monkey, and human testis. Staining for (a) Lin28 and (b) cKIT in mouse testis, (c) Pgp9.5 and (d) cKIT in monkey testis, and (e) and (f) in human testis. Scale bar = 100 μm

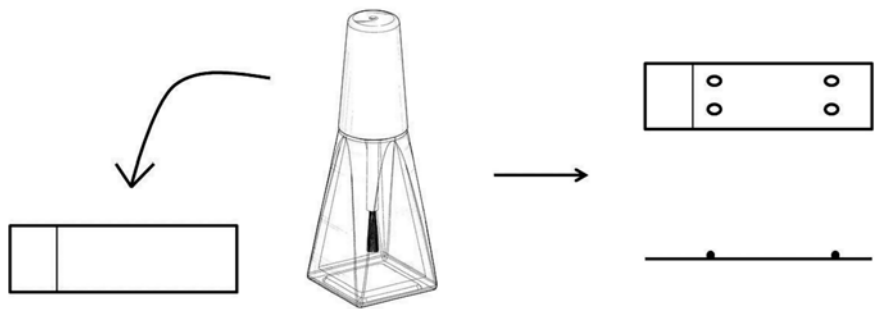


Fig. 4 Raised coverslip glass slides. Four drops of nail polish is put on each corner of a microscope slide to raise the cover glass in order not to smash the sample

Carefully blot on paper towels to remove excess mounting medium. Seal with nail polish.

4. Evaluate with fluorescence microscope suitable to visualize the fluorescent dye conjugated to secondary antibody (e.g., FITC, TRITC) (*see Note 7*).

4 Notes

1. When preparing tubules, all the interstitial tissue should be dissected out as well as possible. This will improve the quality of the tubules, reduce unspecific antibody attachment to interstitial cells, and reduce autofluorescence. Overall staining results are directly related to the timely and careful preparation of seminiferous tubules before the fixation in paraformaldehyde.
2. If the staining quality is not optimal, reducing the fixation time in PFA might improve staining quality.
3. Even though the whole mount tissue can be stored at 4 °C, for optimal results, the tissue should be stored for no more than 3 months.
4. When optimizing an antibody, start with the shorter protocol (Subheading 3.4) and if that does not provide satisfactory staining, use the overnight protocol (Subheading 3.3) for increased tissue permeability and penetration with antibodies.
5. If there is a lot of background, try increasing the dilution of the secondary antibody; often that helps reducing background staining.
6. Instead of Vectashield with DAPI, Hoechst 33342 dye can be added to the secondary antibody and the slides can be mounted with Gelvatol or other mounting media of choice.
7. The slides can be stored at -20 °C for longer period of time to preserve the fluorescence staining.

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Chapter 16

Differentiating the Stem Cell Pool of Human Hair Follicle Outer Root Sheath into Functional Melanocytes

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Abstract

Bench-to-Bedside concepts for regenerative therapy place significant weight on noninvasive approaches, with harvesting of the starting material as a header. This is particularly important in autologous treatments, which use one's bodily constituents for therapy. Precisely the stretch between obtaining therapeutic elements invasively and noninvasively places non-intrusive "sampling" rather than "biopsy" in the center of the road map of developing an autologous regenerative therapy. We focus on such a noninvasively available source of adult stem cells that we carry with us throughout our life, available at our fingertips—or shall we say hair roots, by a simple plucking of hair: the human hair follicle. This chapter describes an explant procedure for cultivating melanocytes differentiated from the stem cell pool of the hair follicle Outer Root Sheath (ORS). In vivo, the most abundant derivatives of the heterogeneous ORS stem cell pool are epidermal cells—melanocytes and keratinocytes which complete their differentiation—either spontaneously or upon picking up regenerative cues from damaged skin—and migrate from the ORS towards the adjacent regenerating area of the epidermis. We have taken advantage of the ORS developmental potential by optimizing explant primary culture, expansion and melanogenic differentiation of resident ORS stem cells towards end-stage melanocytes in order to obtain functional melanocytes noninvasively for the purposes of transplantation and use them for the treatment of depigmentation disorders. Our protocol specifies sampling of hair with their ORS, follicle medium–air interface primary culture, stimulation of cell outgrowth, adherent culture and differentiation of ORS stem cells and precursors towards fully functional melanocytes. Along with cultivation, we describe selection techniques for establishing and maintaining a pure melanocyte population and methods suitable for determining melanocyte identity.

Key words Melanocytes, Hair follicle, Outer Root Sheath, ORS, Stem cells, Skin regeneration, Pigmentation, Vitiligo, Precursors, Differentiation

1 Introduction

The human hair follicle is comprised of the inner and the outer root sheath (IRS and ORS). The inner root sheath gives rise to the hair shaft itself. The ORS is rich in pluripotent, or at least multipotent, stem cells and various precursors that engage in regenerative functions [1–7]. This stem cell niche contains primitive epidermal

leucine-rich repeat containing G protein-coupled receptor-positive (Lgr6⁺) stem cells [8] and it is thus developmentally and in a regenerative sense a forerunner to the comparable niches of the sebaceous gland and interfollicular epidermis [9, 10]. The pluripotent cells originate from embryonic dorsal ectoderm Neural Crest Stem Cells (NCSC) (the dorsal “upper” part of the neural tube) which are able to give rise to most lineages of the adult organism and contribute to most adult tissues [3, 5, 7]. The direct descendants of the NCSC have been named NCSC-like stem cells and they retain their pluripotency to a large extent [3–5]. As far as we know, NCSC-like stem cells have the highest developmental potency among all adult stem cells and display very similar patterns of pluripotent gene expression to that of human embryonic stem cells [3, 5, 9]. The majority of these cells reside in the bulge, the part of the ORS hair follicle proximal to the sweat gland channel interface (the uppermost, isthmal part); nevertheless, they are also present in the mid and proximal part of the ORS. It has been demonstrated by many that NCSC-like stem cells have the potential to give rise to adipocytes [7, 11, 12]; chondrocytes [7]; osteocytes [7]; smooth muscle cells [7]; endothelial cells [13]; cardiomyocytes [14]; neurones, glia [7, 15–18]; melanocytes, keratinocytes [2–4, 8–11, 19–22]; and fibroblasts [15–17]. The most prominent lineages that originate from NCSC-like SC’s of hair follicle ORS are either neural precursors (giving rise to neurons or glia) [7, 15–18] or skin precursors (able to differentiate into keratinocytes, melanocytes, and fibroblasts) [2–5, 8, 9, 11, 17, 19–22].

A certain degree of differentiation perseveres among the cells of the ORS pool and varies along the proximal-distal axis of hair. The lower (proximal) part of the ORS generally comprises cells that are differentiated further than the precursors in the upper (distal) part. Thus, the bulge is considered to be the most pluripotent region of the ORS [3, 5], while the proximal region contains more differentiated cells along with differentiated keratinocytes and melanocytes [2–4, 8, 9, 11, 19–22]. The differentiated cells of the epidermis eventually migrate laterally out of hair follicles for the purposes of epidermal homeostasis and remodelling [20, 23, 24]. The proximal area is also rich in fibroblasts [9, 11]. According to some authors, this part is rich in mesenchymal precursors too [14, 25], at this stage morphologically similar to fibroblasts and therefore difficult to distinguish. The middle region between the bulge and the proximal part is rich in epithelial lineage precursors and presents the most prolific source of keratinocytes [23, 26, 27] and melanocytes [9, 11, 28] although it also harbors mesenchymal potential [14, 25] and gives rise to fibroblasts [9, 11].

This simplification of the ORS developmental variety is not intended to challenge previously substantiated theories regarding the state of the ORS developmental potency or differentiation along the proximal-distal axis of hair. These are described elsewhere in

broad detail and complexity and exceed the scope of this text [10, 29]. Rather, this chapter is dedicated to an optimized noninvasive explant procedure designed to gain as many mature melanocytes from hair follicles as possible in the shortest achievable time by using non-detrimental agents. It relies on the work of Dieckmann et al. described in [28], which is based on studies by Tobin et al. [19]. Nevertheless, our modifications have markedly increased the output of explant cultures, resulting in a cultivation protocol that reproducibly and simply yields 10^6 melanocytes within 4 weeks, and offers an average potential of more than 2×10^6 melanocytes per follicle over a longer period [9, 11, 30]. This is more than sufficient to engineer a melanocyte graft for the purposes of treating depigmentation disorders, commencing with per se harmless sampling in a minimal amount [30].

The procedure focuses on the middle region of the ORS together with the distal isthmal region that interfaces the sweat gland channel (the bulge), since it is able to yield an optimal number of melanocytes with the smallest number of other “contaminating” cell types. The central part of the ORS is the one that can be sampled with the utmost consistency, whereas the distal bulge part, due to its anatomic bond with the sweat/sebaceous gland channel, mostly remains in the epidermis and it is sampled only by chance and even then only partially. The proximal part, which contains already differentiated cells and frequently presents a source of carry-over fibroblasts and MSCs, is removed immediately after sampling [9, 11, 30]. This makes the middle part of the ORS by and large the most important noninvasive source of hair follicle stem cells.

The middle and the semi-distal parts of the ORS together with hair shafts are partially proteolyzed by digestion with Collagenase V in order to loosen the extracellular matrix [9, 30, 31]. The mechanical and intercellular contact inhibition within the ORS is thus reduced and the ORS cells can grow, multiply, and migrate more easily [9, 11, 30].

The pre-digested follicles are further cultivated in medium–air interface conditions, lined up over the nylon mesh of the Transwell® inserts and hereby supplemented with the DLM melanocyte-cultivating medium from the lower side and with a hypoxic gas mixture from the upper side [9, 28, 30]. The cells are allowed to divide and migrate out of the ORS, populate the nylon mesh, and proliferate for 2–3 weeks. Adherent proliferating cells are flooded with medium, and further cultivated until a confluence of 90 % is reached. The Medium–Air Interface primary culture phase ends here. The cells are trypsinized, harvested from Transwell® meshes, and further cultivated adherently in cell culture vessels.

The cells are passaged further upon reaching 70 % confluence of the culture flask, roughly once a week. Doubling rates can differ at this stage, depending on the donor specifics.

At this point, the culture is still in its primary phase and typically also contains stem cells and non-melanocytic precursors [9, 28, 30]. This pool of precursor cells is used for giving rise to melanocyte precursors and finally to melanotic melanocytes.

In order to avoid differentiation into end products other than melanocytes, the adherent culture is subjected to two selection methods. The initial selection method is treatment with Geneticin, a translational blocker [32] that primarily affects quickly dividing cells—in this case, keratinocytes and fibroblasts, along with proliferating precursors. They are all selected away on the basis of their intensive need for protein synthesis, which is clogged up by Geneticin. Melanocyte precursors and melanocytes are, as a rule, slowly dividing cells and they are thereby spared from the Geneticin effect, since they are less sensitive to changes in protein synthesis. After a 48 h selection treatment, the remaining living cells in culture will be almost exclusively melanocytes [9, 11, 30].

The second selection method, differential trypsinization, maintains the high number of melanocytes in culture and eliminates other cells types in each passage. Melanocytes weakly adhere to the polystyrene surface of the cell vessel and are prone to retracting their dendrites and quickly detaching upon being exposed to trypsin. Keratinocytes and fibroblasts adhere more firmly and are less inclined to changing their habitus during trypsinization. If the trypsinizing conditions are maintained suboptimally, in our case at room temperature instead of 37 °C and over a shorter exposition time of 4 min, most of melanocytes will detach whereas most of the keratinocytes and fibroblasts will still remain adherent. The detached melanocytes in the supernatant are taken into the next passage and placed into a new cell flask. If applied at each passage, this method ensures a negligible-to-zero content of keratinocytes or fibroblasts and keeps the melanocyte culture pure [9, 11, 26, 27, 30].

From passage 3 onwards, the cells are tested for purity and characterized on the basis of morphology, marker expression and melanin production. Typically, as soon as the cells are morphologically correct and present in sufficient numbers, they are tested by qRT-PCR for expression of genes typical for melanocytes—paired box 3 (PAX3), premelanosome glycoprotein 100, GP100 (PMEL), tyrosinase (TYR), microphthalmia-associated transcription factor (MITF) and for the absence of pluripotency gene expression (Table 1). The expression of the following proteins is tested by immunofluorescence: tyrosinase, the key enzyme in melanin production and glycoprotein100 variants NKI-beteb and HMB45 (gp100, resident protein in melanosomes, the secretion vesicles packed with melanin).

The marker-positive cells are further tested for the activity of tyrosinase, tyrosinase-related protein 1 and 2 (TRP-1, TRP-2) by the L-DOPA test and for melanin content. The cells positive in all tests are regarded as fully functional.

Fully graded cells are cryopreserved and archived.

Table 1
Genes of interest

Full name	Abbreviation	Marker
Nanoghomeobox	NANOG	Pluripotency
POU class 5 homeobox 1, OCT4	POU5F1	Pluripotency
Zinc finger protein 42 homolog, REX1	ZFP42	Pluripotency
Lin-28 homolog A	LIN28A	Pluripotency
v-Kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	KIT	Pluripotency
Paired box 3	PAX3	Melanotic
Premelanosome protein, GP100	PMEL	Melanotic
Tyrosinase	TYR	Melanotic
Microphthalmia-associated transcription factor	MITF	Melanotic
Hypoxanthinephosphoribosyltransferase 1	HPRT1	Housekeeping
Mitochondrial ribosomal protein L15	MRPL15	Housekeeping

2 Materials

2.1 Cell Lines

1. Normal Human Epidermal Melanocytes (NHEM, Promocell). Primary culture of melanocytes isolated from human skin; used as a positive control for melanocyte marker expression. The NHEM are maintained in the Promocell Melanocyte Growth Medium M2 at 37 °C, 5 % CO₂, under normoxic conditions.
2. HUMORS—Human Melanocytes developed from ORS (melanoblasts, amelanotic and melanotic melanocytes). The cells are maintained in a DLM (DermaLife) medium, at 37 °C, in hypoxic conditions (5 % O₂, 5 % CO₂). From passage 4 on, the tested cells are cryopreserved and stored at the cell bank.
3. Human fibroblasts (www.atcc.org) are used as a positive control for CD90 expression during the purity testing and as a negative control for tests of melanocyte identity. The cells are maintained in a DMEM-based Fibroblast medium at 37 °C, 5 % CO₂, in normoxic conditions.

2.2 Technical Equipment

1. Incubator (37 ± 1 °C), humidified, normoxic 5 ± 1 % CO₂/air.
2. Incubator (37 ± 1 °C), humidified, hypoxic 5 ± 1 % O₂/5 ± 1 % CO₂/air.
3. Laminar flow clean bench (standard: “biological hazard”).
4. Phase contrast microscope.
5. Fluorescence microscope.

6. Clamp surgical forceps.
7. Tissue culture disposables: Cellstar 35 mm, T-flasks 25 cm², T-flasks 75 cm², (routine culture), 6-well tissue culture plates, 24-well tissue culture plates, 96-well tissue culture plates with flat bottom, 96-well tissue culture plates with U-bottom, 70 µm Cell Strainer, Chamber Permanox Slide, 8-well tissue culture plates, 24 mm Transwell® with 0.4 µm Pore Polyester Membrane Insert with 6-well.

2.3 Reagents

Media.

1. Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine, glucose, and NaHCO₃.
2. Derma Life Melanocyte Medium (DLM) Basic Medium (CellSystems®).
3. FrostaLife (Freezing medium) (CellSystems).
4. Melanocyte Growth Medium M2 (PromoCell GmbH).
5. Cryo-SFM Freezing medium (PromoCell GmbH).
6. Fetal Bovine Serum.

Antibiotics.

7. Penicillin/Streptomycin solution.
8. Gentamicin solution.
9. Geneticin solution G-418 Sulfate.
10. Amphotericin solution.

Cell biological reagents.

11. Phosphate buffered saline (PBS) without Ca⁺⁺ and Mg⁺⁺ pH 7.4,
12. PBS with Ca²⁺ and Mg²⁺ pH 7.4.
13. HEPES buffer saline.
14. Gelatin.
15. Paraformaldehyde.
16. Albumin bovine serum (BSA).
17. Goat serum (PromoCell).
18. Saponin.
19. Fluoromount™-G™.
20. Ethanol (analytical grade).
21. Methanol 99.9 %.
22. NaOH.

Antibodies, cell dyes.

23. HMB45 mouse monoclonal antibody (Abcam).
24. NKI-beteb mouse monoclonal antibody (Abcam).

25. Tyrosinase mouse monoclonal antibody (Abcam).
 26. Anti-Mouse IgG (h+L), made in goat biotinylated 1.5 mg, (BIOZOL Diagnostica).
 27. Alexa Fluor 594 goat anti-mouse IgG (H+L) 2 mg/ml.
 28. Streptavidin Alexa Fluor 488 conjugate 2 mg/ml.
 29. Streptavidin Alexa Fluor 594 conjugate 2 mg/ml.
 30. CD90/Thy-1-Hybridoma-S/N mouse primary antibody AS02 [33, 34].
 31. DAPI 10 mg.
 32. Qnuclear Deep Red Stain.
 33. Trypan Blue.
- Enzymes, substrates.
34. Trypsin/EDTA solution 0.04 %/0.03 %.
 35. Trypsin neutralizing solution.
 36. L-DOPA (3,4-Dihydroxy-L-phenylalanine).
 37. Collagenase Type V.

2.4 Preparations

2.4.1 Culture Media

1. Melanocyte Growth Medium M2 is used for cultivating the human epidermal melanocyte NHEM cell line. For 500 ml of Melanocyte Growth Medium M2, add the entire content of the M2 supplement mix to the basic medium. Melanocyte Growth Medium M2 is also available as a ready-to-use kit.
2. DLM Melanocyte Medium is used for cultivation of the precursors of human melanocytes derived from the human hair root and their subsequent differentiation into functional melanocytes. DLM Medium base (48 ml) with a low serum content is used with addition of L-Glutamine 1.5 ml (6 mM), $[Ca^{2+}]$ DLM 50 μ l (0.2 mM), insulin 50 μ l (5 μ g/ml), vitamin C 50 μ l (50 μ g/ml), epinephrine 50 μ l (1.5 μ g/ml), Stimel™ factor 500 μ l (1:100), Gentamicin 250 μ l (50 μ g/ml) (*see Note 1*).
3. Fibroblast Medium. Formula for 50 ml of fibroblast medium: DMEM Basal medium w/o Ca, Mg or L-Glu 44 ml, Fetal Calf Serum 5 ml (10 %), L-Gln 0.5 ml (2 mM), Penicillin/Streptomycin 0.5 ml (1 %).

3 Methods

3.1 General Advice for Cell Culture Before Starting

1. Use freshly prepared reagents and medium. Refrigerate the medium optimally no longer than 1 week and by no means longer than 1 month.
2. Preheat solutions and medium for 30 min at room temperature or in a 37 °C water bath for 10 min.

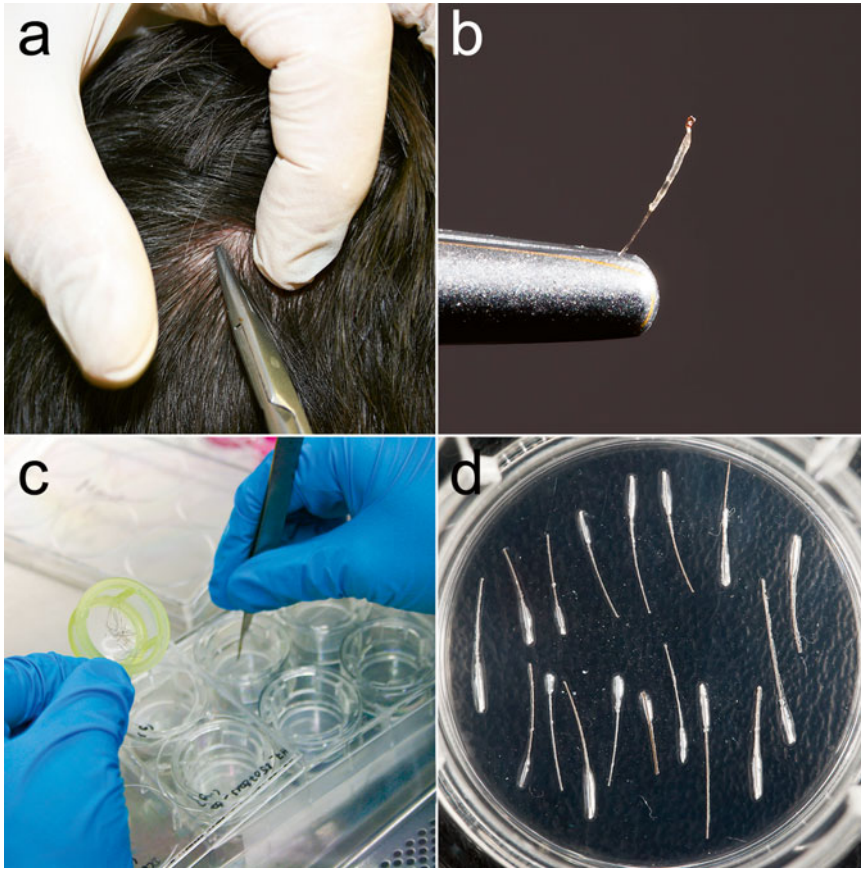


Fig. 1 Sampling and preparation of hair follicles. **(a)** Plucking hair using a pair of forceps. **(b)** Freshly plucked follicle. **(c)** Lining up washed and digested follicles (in cell strainer, *left*) on Transwell® inserts (*middle*). **(d)** Follicles lined up on Transwell® inserts

3. Avoid prolonged exposure to light (e.g., under the microscope) when using DLM medium. Protect the medium bottle from light (or use the DLM-base protective foil bottles provided).
4. Check your cultures with a microscope before changing the medium. Check regularly and carefully for impurities in the culture. Apply special caution when working with primary cultures.

3.2 Explant Primary Culture of Human Outer Root Sheath Cells from Hair Roots

3.2.1 Sampling

1. Prepare disinfected round ending forceps, fine forceps, surgical scalpel, two clean petri dishes per proband, $d = 5$ cm, add 5 ml PBS to each dish. This part of primary culture is non-sterile.
2. Lift the proband's hair with your fingers to expose the roots. Place the lower jaw of the open forceps underneath the hairs that you are about to pluck out. Choose five hairs in a single row. Place the forceps sideways along the line of the row and as close to the root as possible. Hold the hairs tight with the forceps, clamp and pull in the direction of hair growth (Fig. 1a).

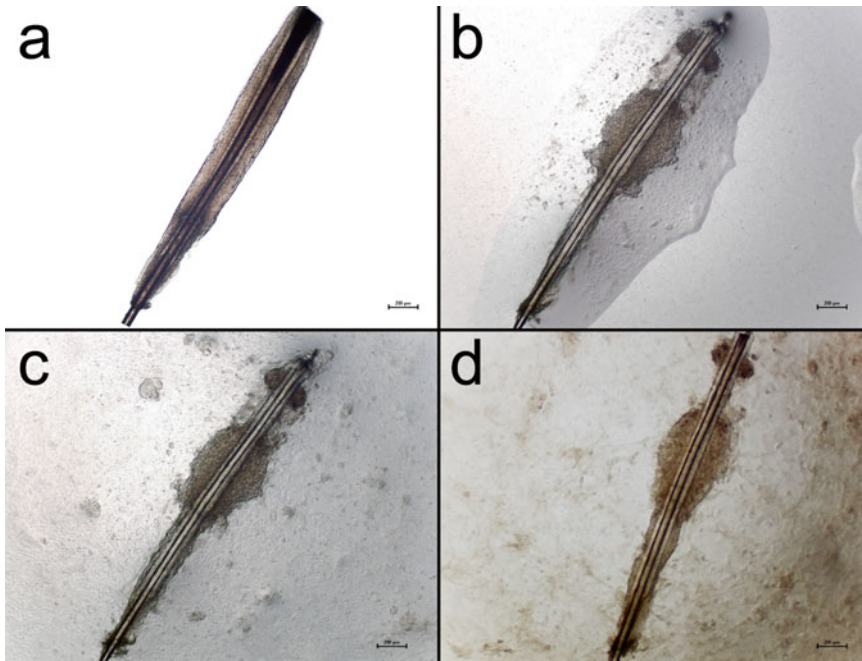


Fig. 2 Medium–air interface primary culture of the Outer Root Sheath. (a) Medium–air interface, day 0. Hair follicle with a severed proximal end. (b) Medium–air interface, day 7. Outgrown hair follicle with adjacent migrated cells on the Transwell® insert mesh. (c) Medium–air interface, day 14. Flooded hair follicle. Dividing cells forming colonies are visible next to the follicle. (d) Medium–air interface, day 28. The cells on the Transwell® insert mesh are confluent

3. Check if the follicles have been pulled together with the extracted hairs by holding them against a light source. It will take some effort to spot the follicles and they will appear as a transparent, narrow gelatinous envelope around the proximal (root) part of the hair shaft. Keep track of the follicle number (Fig. 1b).
4. Shorten the hair from the distal side, leaving 2 cm as a “handle” for the hair follicle (Fig. 1b). Place the prepared follicles in PBS. Plucking and the freshly harvested follicle are shown in Fig. 1 (see **Note 2** for hints on follicle sampling).
5. Follicle preparation: Place the petri dish with PBS and hair roots under a binocular microscope, with another petri dish containing fresh PBS next to it. Hold the hair root by the hair part with fine forceps and cut off the proximal portion of the follicle (the lower 20 % in length, Fig. 2a). Place the shortened follicle into the second petri dish with fresh PBS.
6. The rest of the preparation steps should be completed in sterile conditions.

3.2.2 Primary Antibiotic Treatment

1. Prepare 5.5 cm petri dishes and 10 ml PBS with Gentamicin solution (50 µg/ml) and Amphotericin (10 µg/ml). Add 2 ml of the antibiotic solution to each dish.

2. Collect the shortened follicles from the petri dish and place them on a 70 μm cell strainer (Fig. 1c).
3. Submerge the strainer with follicles into each petri dish with Gentamicin/Amphotericin for 2 min. Agitate gently.
4. Wash by dipping the strainer insert into a petri dish with PBS.

3.2.3 Collagenase V Treatment

1. Aspirate the PBS and add collagenase V solution. Leave the roots submerged in the collagenase V solution to digest for 10 min at 37 °C.

3.2.4 Mounting the Primary Culture of Hair Roots

1. Line up the hairs with pre-digested follicles onto a Transwell® mesh insert as shown in Fig. 1c, d. Ten follicles per mesh are sufficient. Use the same orientation of the roots and line them up parallel to one another. This helps when monitoring the follicles, which you will be doing often in the next weeks (*see Note 2*).
2. Place the Transwell® insert into uncoated 6-wells. Add 1 ml of DLM medium underneath the inserts. The medium should make contact with the bottom of the insert. This creates the medium–air interface (*see Note 4*).
3. Add 2 ml of PBS into the pockets between the wells, to maintain humidity.
4. Place the well into a hypoxic incubator supplied with 5 % O_2 /5 % CO_2 (*see Note 5* for alternative solutions to hypoxic conditions). The hair roots can be left unattended and supplied with the same medium during the first week of cultivation. Inspect regularly for contamination. Take care not to disturb the wells mechanically since the follicles could detach.

3.2.5 Maintaining the Primary Culture of Hair Root ORS Cells

1. After not later than 1 week of incubation, observe and photograph the follicles (Fig. 2). They should assume a typical swollen morphology, with first signs of the ORS outgrowth, encapsulated in a drop of medium moisture. The first single cells that have migrated from the follicles should be visible on the mesh next to the follicles (Fig. 2b).
2. Aspirate the medium from the well beneath the Transwell® insert using a Pasteur pipette and a vacuum pump. Do not remove the Transwell® insert. Use one of the three openings at the inner ring of the Transwell® insert to reach the bottom of the well with the medium.
3. Add 1 ml fresh DLM medium to the bottom of the well through the inner ring opening. Use 1 or 2 ml pipettes for this purpose. Change the medium every 2 days.
4. Observe the growth of the ORS every 2 days. After 2–3 weeks, the migrating cells should be clearly visible alongside the follicle. Some of them will be coating the mesh in a single layer and

some will form colonies (Fig. 2b, c) (*see* **Notes 3, 4, 6–8** for monitoring, maintaining, and documenting your Medium–Air Interface follicle culture).

3.2.6 Flooding: Expanding the Primary Culture of Hair Root ORS Cells

1. Upon visible migration and proliferation of the cells that have left the ORS (not later than 4 weeks post-biopsy), a rough monolayer should have formed around the hair roots (Fig. 2d). Document by photographing. This is a good point to stop the medium–air interface cultivation and flood the cells with medium.
2. Add 1 ml of the DLM medium to the upper side of the mesh insert. Leave the cells to proliferate until they are nearly confluent. Document frequently in the event of rapid growth (Fig. 2) (*see* **Notes 7 and 8** for medium–air interface and the flooded follicle/ORS culture particularities in more detail).

3.2.7 Adherent Primary Culture of ORS Cells

Starting the primary culture of adherent ORS cells.

1. At the point of near confluence, and optimally 3 and not later than 6 weeks post-sampling (Fig. 2d), document and trypsinize the cells from the mesh insert: Aspirate the medium, wash the cells once with HEPES buffer, aspirate the buffer and add 2 ml of 0.04 % trypsin/0.03 % EDTA.
2. Trypsinize the culture in the insert together with hairs for 5 min at 37 °C and for another 5 min at room temperature. Prolong if necessary.
3. Add 2 ml of trypsin inhibitor.
4. Pipette up and down until the cells detach from the Transwell® mesh.
5. Transfer to a 15 ml Falcon tube and add 5 ml of PBS. Pipette up and down until the cells are suspended and no clumps are visible.
6. Count the cells using the Neubauer hemocytometer.
7. Centrifuge the cell suspension at $220 \times g$ for 5 min.
8. Discard the supernatant cautiously, using the Pasteur pipette.
9. Resuspend the pellet in 1 ml of DLM medium. The choice of the cell vessel depends on your harvest. A rule of thumb: If you have obtained 80,000 cells or less, seed them into a 6-well (10 cm^2); for 80,000–300,000 cells, use a T25 flask (25 cm^2) and for more than 300,000 cells, use a T75 flask (75 cm^2). The culture ware should be non-coated.
10. Depending on the cell count, transfer the entire 1 ml cell suspension into a 6-well or add 4 ml DLM medium and transfer into a T25 cell culture flask, or add 11 ml medium and transfer into a T75 flask.

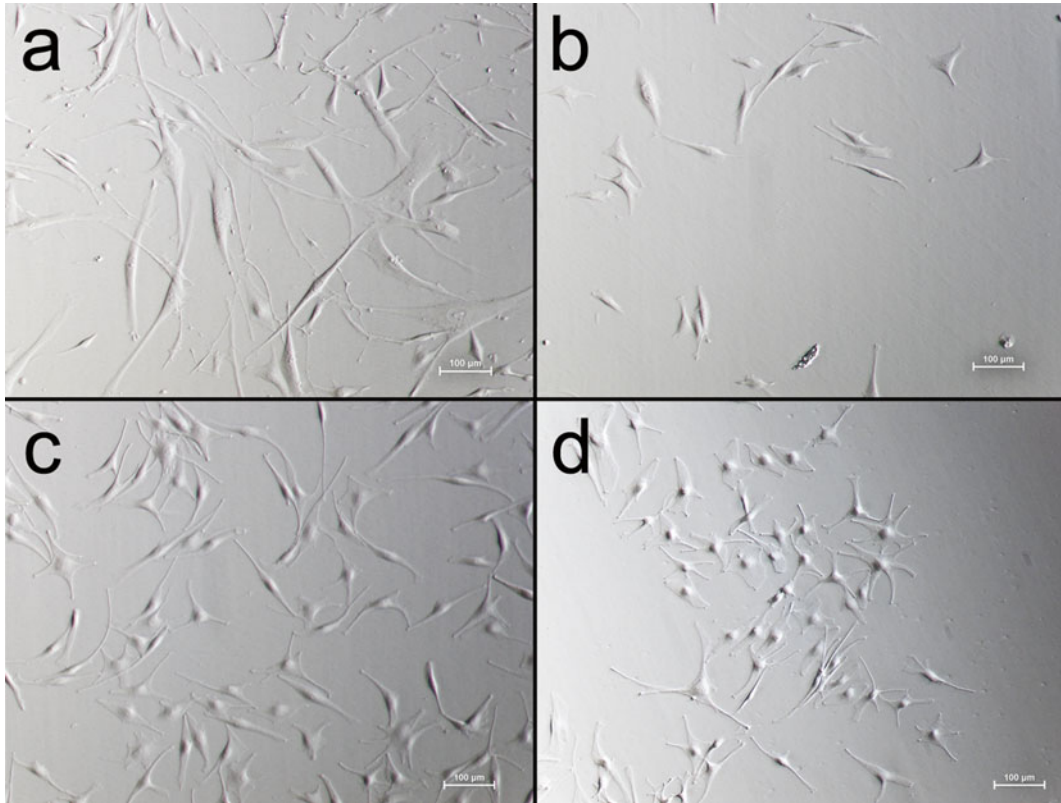


Fig. 3 Adherent culture of the Outer Root Sheath cell pool. **(a)** Adherent culture, p0. Heterogeneous cell population displaying various morphologies. **(b)** Adherent culture, p1. Homogenous cell population remaining after Geneticin selection. The cells are still mostly bidendritic. **(c)** Adherent culture, p3. Homogenous population of melanocyte precursors and HUMORS maintained by differential trypsinization. Tri- and poly-dendritic cells are visible. **(d)** Adherent culture, p5. HUMORS. Most of the cells are displaying standard melanocyte morphology with several dendrites

11. Place the cell vessel with the seeded cells in the hypoxic incubator supplied with 5 % O₂, 5 % CO₂ mix. Cultivate at 37 °C.
12. Change the medium every 2 days.

3.2.8 Maintaining the Primary Culture of ORS Adherent Cells

1. Observe the cells every 2 days for adherence, morphology, and confluence. Document with photographs (Fig. 3a–d).
2. At 80 % confluence, passage and re-seed at a 1:2 ratio if you are planning quick expansion or a 1:4 ratio if you aim to subculture for a longer time.
3. Passage using differential trypsinization (4 min at room temperature, using the detached fraction of cells for the next passage) as described just below among the selection methods (*see* **Note 11**).

*3.2.9 Selecting
the Melanocytes
from the Primary Culture
of ORS Cells*

1. Commonly, fibroblasts and keratinocytes also occur in the primary culture set up from follicle ORS (Fig. 3a). Fibroblasts assume a spindle-like morphology and the keratinocytes are pebble-like, both clearly differing from the melanocyte habitus (*see Note 10*). In order to get rid of the fibroblasts and keratinocytes, we recommend two clear-cut selection methods (*see Note 11*).
2. Treat with 50 µg/ml Geneticin in DLM medium for 48 h. The translation interference of the Geneticin will affect more active and quickly proliferating cells, in this case fibroblasts and keratinocytes, since their demand for protein synthesis is far higher than that of slowly dividing melanocytes.
3. After the Geneticin treatment, leave the cells to recover for no less than 24 h in DLM medium. Some more cells will die. The vast majority of the surviving cells will be melanocytes or melanocyte precursors (Fig. 3b). Change the medium. Leave to proliferate until they are 80 % confluent. Apply Geneticin treatment once at the beginning of the adherent culture (in p1).

*3.2.10 Differential
Trypsinization*

1. Maintain the selection by differential trypsinization. The melanocytes are less adherent than fibroblasts and keratinocytes, and they will detach even under suboptimal conditions for trypsin action—room temperature and shorter time.
2. Extract the medium with a Pasteur pipette connected to the vacuum pump.
3. Wash cells by gentle agitation twice with HEPES buffer.
4. Discard the washing solution under vacuum.
5. Add room temperature pre-warmed Trypsin/EDTA solution to the monolayer (1.5 ml/T75 flask; 0.5 ml/T25, 300 µl/6-well).
6. Incubate at room temperature for 4 min.
7. By now, the melanocytes will have detached. Check using a microscope: detached melanocytes float around as the vessel is moved back and forth; fibroblasts and/or keratinocytes are adherent.
8. Neutralize with an equal amount of Trypsin neutralizing solution.
9. Resuspend the cells from the bottom of the flask by thoroughly pipetting up and down and washing the entire surface with a jet from the pipette.
10. Collect cells in a 15 ml tube.
11. Pipette up and down until no cell clumps are visible.
12. Centrifuge the cell suspension at $220\times g$ for 5 min.
13. Carefully discard the supernatant above the pellet using a Pasteur pipette.

14. Resuspend the cells in 5 ml DLM medium by pipetting up and down 5–10 times.
15. Split the cells in a 1:4 ratio using 1.5 ml of single-cell suspension and 4.5 ml of DLM medium for a T25 flask, or 3 ml of suspension with 9 ml DLM for a T75 flask. This typically results in 120,000 seeded cells per T25 flask or 360,000 cells per T75.
16. Transfer the cells and the medium into the flask.
17. Cultivate in a hypoxic incubator (5 % O₂, 5 % CO₂) and cultivate at 37 °C.
18. Change the medium every 2 days.
19. Apply further differential trypsinization at each passage. Maintained cells will be in stages that precede fully differentiated melanocytes and they will have a characteristic bipolar or tripolar pre-star-like morphology, with protrusions (Fig. 3c) (*see Note 9–11*).

3.2.11 Routine Culture of Human Melanocytes from the ORS (HUMORS) and Their Precursors

1. In an adherent culture, HUMORS routinely grow as a monolayer in uncoated culture flasks at 37 °C in a humidified hypoxic atmosphere of 5 % O₂, 5 % CO₂.
2. The cells should be examined on a daily basis using a phase contrast microscope. Typically, they will display three or more dendrites (Fig. 3d).
3. Change the DLM medium every second day.
4. Melanocyte precursors are routinely passaged at a cell confluence of 70–80 %, which corresponds to a density of $0.4\text{--}0.5 \times 10^6$ cells/T25 flask. Between P3–P13, their doubling time is 1 week.
5. Cells should be maintained for no longer than 18 passages, which roughly correspond to 4–5 months of cultivation at the rate of 1 passage per week. Cryopreserve the HUMORS in FrostaLife™ freezing medium.

3.2.12 Normal Human Epidermal Melanocyte (NHEM) Subculture

1. Normal Human Epidermal Melanocytes are routinely passaged at a cell confluence of 70–80 %, which corresponds to a density of 0.4×10^6 cells/T25 flask. It is usually sufficient to split them at a 1:4 ratio once a week. Trypsinize for 4 min at room temperature. Splitting the 80 % confluent T25 flask with NHEM 1:4 typically results in 100,000 cells per T25 flask.
2. Incubate the NHEM at 37 °C in normoxic conditions. Replace the medium of the subcultures every 2 days. The cells should be maintained for no longer than 15 passages after thawing, which roughly corresponds to 4 months at a rate of 1 passage per week. Use Cryo-SFM freezing medium for cryopreserving.

3. The passaging procedure is the same as the differential trypsinization and valid for all cultures described in this chapter, except for **steps 5, 6, and 7**.
4. Incubate for 4 min at room temperature for HUMORS and NHEM, for 5 min at 37 °C for or fibroblasts.
5. If you still see adherent cells in the fibroblast or melanoma culture, trypsinize for one more minute at 37 °C, then recheck.
6. Neutralize with an equal amount of Trypsin neutralizing solution. Collect the supernatant with detached HUMORS or resuspend the NHEM, fibroblasts or melanoma cells from the bottom of the flask by thoroughly pipetting up and down and washing the entire surface with the jet from the pipette.

3.2.13 *Characterizing the Melanocytes in ORS Cell Culture*

1. In order to know what exactly you have at hand, the melanocyte culture should be characterized for melanocyte morphology, the marker expression characteristic for melanocytes and production of melanin. Melanocyte identity is determined by the expression of tyrosinase and two variants of glycoprotein 100 (gp100): HMB45 and NKI-beteb. Melanotic melanocyte status can be determined by the enzymatic activity of the tyrosinase DOPA-converting enzyme set, tyrosinase-related protein 1 and 2 (TRP-1, TRP-2) by observing and quantifying L-DOPA conversion to melanin, and by quantifying the melanin content of the cells.
2. The presence of fibroblasts can be determined by CD90 (Thy-1-Hybridoma-S/N) expression; the fibroblasts will express CD90 clearly (CD90+) whereas melanocytes will not (CD90-).
3. Use the cells from the same passage for characterization. For all tests, set up fibroblasts and NHEM as controls and HUMORS to be tested.

3.2.14 *Immunofluorescence Analysis for CD90/Thy-1-Hybridoma-S/N*

1. Prepare glass 8-slot chamber slides by incubating with sterile 0.1 % gelatine-PBS for 15 min. Lift the lid and discard the gelatin with a Pasteur pipette. Leave the chamber slides without the lid to dry on a clean bench for 15 min.
2. Seed no less than 5,000 cells per chamber onto glass chamber slides and cultivate for another 2 days, fibroblasts normoxic in fibroblast medium, HUMORS hypoxic in DLM, NHEM normoxic in M2 medium, at 37 °C.
3. Discard the medium and wash once with PBS.
4. Fix the cells. For CD90: use 4 % paraformaldehyde (PFA) for 8 min at room temperature; discard the PFA subsequently. For tyrosinase, NKI-beteb, and HMB45: use ice-cold methanol at -20 °C for 10 min, and air-dry subsequently.
5. Wash the cells once with PBS for 10 min.

6. Block. For CD90 use 2 % BSA-PBS for 60 min RT. For tyrosinase, and NKI-beteb incubate with 200 μ l of 0.1 % saponin-PBS at room temperature for 10 min. For tyrosinase, NKI-beteb, and HMB45 block with 150 μ l of 20 % goat serum-PBS-0.1 % Tween for 1 h at room temperature.
7. Prepare 150 μ l of primary antibody solution. For CD90 mouse-generated monoclonal AS02 CD-90 antibody 1:3 in 2 % BSA-PBS; for tyrosinase, NKI-beteb, and HMB45 the corresponding antibody 1:50 in 2 % BSA-PBS-Tween.
8. Incubate the cells with the antibody for 2 h at room temperature or overnight at 4 °C with agitation and protected from light. Placing the slides in a small chamber with antibody solution on a rocker and covering with simple plastic vessel will suffice.
9. After 2 h, discard the primary antibody solution and wash the cells twice with 200 μ l of PBS for CD90 and PBS-0.1 % Tween for tyrosinase, NKI-beteb, and HMB45, 5 min with agitation.
10. Prepare a secondary antibody solution. For CD90: goat-anti-mouse-IgG-Alexa594 (1/300) and DAPI (1/1,000) in 2 % BSA-PBS. For tyrosinase, NKI-beteb, and HMB45: goat-anti-mouse-IgG-biotin (1/400) in 2 % BSA-PBS-T.
11. Incubate with the secondary antibody for 1 h at RT. Discard the secondary solution and wash the cells. For CD90: 3 $\times$ with PBS, discard PBS and shortly air-dry. For tyrosinase, NKI-beteb, and HMB45: wash twice with 200 μ l of PBS-0.1 % Tween.
12. Prepare 150 μ l of streptavidin solution: SA-Alexa 488 (1/400, 5 μ g/ml) and DAPI (1/1,000) in 2 % BSA-PBS-T.

Steps 13–15 apply only to tyrosinase, NKI-beteb, and HMB45.

13. Incubate the cells with SA-Alexa-DAPI solution for 1 h at room temperature.
14. Discard the solution; wash twice with 200 μ l of PBS-0.1 % Tween and air-dry briefly.
15. Mount by thickly covering with Fluoromount™ liquid. Use a glass rod to apply the Fluoromount™ liquid and gently place a glass coverslip over the liquid layer. The coverslip should not slide in any direction. Tap lightly over the coverslip in case of air bubbles. If the air bubbles persist, remove the coverslip, and repeat the mounting by removing the bubbles with a 10 μ l pipette tip, adding more Fluoromount™ and re-covering.
16. Let the mounted slip dry and harden for 24–48 h at 4 °C.
17. Analyze by fluorescence microscopy. Fibroblasts will display CD90 signal, NHEM and HUMORS will display tyrosinase, NKI-beteb, and HMB45 signals.

3.2.15 Melanin- Production in Melanocytes

1. Detach the cells with trypsin/EDTA solution for 4 min at room temperature. Pipette vigorously to form a single-cell suspension.
2. Count the harvested cells to determine the exact number of the cells to be tested.
3. Wash once in 5 ml PBS.
4. Pellet the cell suspension by centrifugation at $220 \times g$ for 5 min.
5. Discard the supernatant and resuspend the cell pellet in 1 N NaOH.
6. Keep at 60 °C for 3 h with gentle agitation (use a water bath).
7. Pellet and transfer the supernatant into a flat-bottom 96-well.
8. As control, set up the following array of dilutions of synthetic melanin for the melanin standard curve in a flat bottom 96-well plate: 0/3.125/6.25/12.5/25/50/100 µg/ml, one concentration per well.
9. Measure the optical density/extinction of the samples and controls using a plate reader at 475 nm wavelength. Retrieve the melanin concentration by linear regression comparing the extinction values of the samples with the melanin concentration standard curve.
10. Calculate the total amount of melanin in the culture using (concentration $\times$ volume).
11. Express the melanin content as melanin µg/culture, or ng/cell using the initial total number of melanocytes in the culture.

3.2.16 L-DOPA Reaction

Visual L-DOPA test (qualitative)

1. Cultivate the NHEM for 72 h on glass slides coated with gelatine.
2. Wash the cells 2 $\times$ with 0.1 M PBS pH 7.2 (0.1 M PO₄-buffer (pH 7.2) and 120 mM NaCl).
3. Fix the cells for 30 s in acetone/methanol (1:1) at -20 °C.
4. Wash the cells 2 $\times$ with 0.1 M PBS pH 7.2.
5. Incubate the cells for 5 h at 37 °C in 0.1 M PBS pH 7.2 containing 5 mM of freshly prepared L-DOPA. Change L-DOPA solution three times in the course of the 5-h incubation. A separate sample treated with PBS without L-DOPA should be used as control.
6. Wash the cells 2 $\times$ with 0.1 M PBS pH 7.2.
7. Incubate the cells with Nuclear Fast Red for 10 min.
8. Discard the Nuclear Fast Red solution and rinse cells 2–3 min with water. Gently agitate.
9. Discard the water and briefly air-dry the slide.

10. Mount with Fluoromount™ as described in the immunofluorescence protocol above.
11. Observe the cells through a microscope. The melanocytes incubated with L-DOPA will appear darker. Dark vesicles—melanosomes—will be visible, stretching along the dendrites towards the dendrite ends. Reddish nuclei will be visible.

3.3 Gene Expression Analysis

Gene expression is quantified by means of qRT-PCR. Genes of interest are shown in Table 1.

3.3.1 RNA Extraction

Harvest 100,000 cells by trypsinization and extract RNA with RNeasy® Plus Universal Kit from Qiagen®. Elute RNA with 30 µl RNase-free water. Determine the RNA concentration with a nanophotometer.

3.3.2 cDNA Synthesis

1. Use QuantiTect® Reverse Transcription Kit from Qiagen® to transcribe cDNA to 50 ng/µl. Remove genomic DNA contaminations in the purified RNA samples with gDNA Wipeout Buffer.
2. Reverse-transcribe the samples using a master mix containing oligo-dT and random primers and Quantiscript® Reverse Transcriptase containing RNase inhibitor and dNTPs.

3.3.3 Quantitative Real-Time PCR

1. The QuantiFast™ SYBR® Green PCR Kit from Qiagen® provides rapid real-time quantification. The components of the ready-supplied Master Mix are: HotStarTaq® Plus DNA Polymerase, QuantiFast SYBR Green PCR Buffer, dNTP mix and ROX passive reference dye for fluorescence normalization.
2. Prepare the reaction mix in MicroAmp™ optical 96-well PCR plates as shown in Table 2.
3. Transfer the 96-well PCR plate into the Applied Biosystems® 7500 cycler. The Table 3 depicts real-time cycler conditions used in this protocol.

Table 2
qPCR-Reaction setup

Component	Volume/reaction	Final concentration
2× QuantiFast SYBR Green PCR Master Mix	12.5 µl	1×
Primer	2.5 µl	1 µM
Template cDNA	1 µl	50 ng/µl
RNase-free water	9 µl	
Total reaction volume	25 µl	

Table 3**Real-time cycler conditions, Qiagen® QuantiFast™ SYBR® Green PCR Kit**

Step	Time	Temperature (°C)	Notes
PCR initial activation	5 min	95	HotStarTaq Plus DNA polymerase activated
Two-step cycling			
Denaturation	10 s	95	
Combined annealing/extension	30 s	60	Fluorescence data collection
Number of cycles	40		

4. Use Applied Biosystems® 7500 v2.0.6 data analysis software. Normalize the gene expression against the expression of house-keeping genes hypoxanthine phosphoribosyltransferase 1 (HPRT1) and mitochondrial ribosomal protein L15 (MRPL15).
5. Calculate relative quantification using the ΔCT method—the mean ratio between the amount of target (different marker genes) and the amount of reference genes (housekeeping genes) (*see* **Note 12**).

4 Notes

1. All supplements for the media are provided as separate solutions. They are stored in aliquots at 4 or -20°C according to the manufacturer's instructions. DLM full medium is also available as a kit. The medium should be used within 1 week, stored at 4°C .
2. Collecting follicles: Habitually, spring and summer are better seasons for sampling since anagen follicle content is high at this time. Precisely for the reasons of anagen index, young, healthy, and sporty probands are preferred for sampling, even though this rule is not undisputed—we have met our share of party-loving smokers with dyed hair whose follicles produced prestigious explant cultures. Instruct the hair donors NOT to wash their hair at least 24 h prior to plucking their hairs. Also, no gel or hair spray should be used within that time. Follicles sufficiently oiled by one's own sebum encounter less friction against the follicle cup. Furthermore, the presence of oils makes the walls of the cup more elastic and the follicles are extracted far more easily. Also, loss of follicle material is reduced. This, to all appearances a ridiculous precondition, could be an important optimization step of your protocol. It visibly yields more follicle material and with a bit of luck,

greasy hair could even help yield a part of the bulge, which as a rule remains obstructed between the pilliary muscle and the sebaceous gland channel and remains in the follicle cup. On the other hand, both the washed hair and the surrounding skin are dryer and stiffer, even more so if gels or sprays are used. This makes the follicle cup constricted and jams the follicles. Dry hair tends to snap under such conditions, resulting in a lot more hair being pulled out than follicles, even at a rate of 1:4. Be careful not to pinch the scalp skin while plucking; if you do, the probands will never allow you come near their temporal region again. To ensure painless sampling, the extracting motion should be swift but not forceful (pull using your wrist), and the hair along with the follicles should come out within a single movement.

3. Inspection of outgrowth: Typically, one should check the follicle culture on day 1, day 2, day 4, and day 6. The first two days are crucial for the sterility of the culture and if you do not see contaminations by day 4, there is cause for optimism. If you see fungal contamination at this stage, discard the culture. After 1 week, the first single cells will be visible on the mesh next to the follicles. Document them. Observed from above, the follicles should be wet as if they were enclosed within a single drop that stretches along the follicle line. This ensures that the follicles remain moist and nurtured, but richly exposed to the gas mixture due to a thin layer of liquid that covers them. There will be follicles without outgrowth but do not be demoralized by this, it is normal that sometimes a rather large portion of follicles remain idle. This depends on the amount of follicles in the anagen phase.
4. Variations in cell ware for a medium–air interface: When setting up the medium–air interface, one can of course use different inserts and 6-well plates. Please note that not all inserts and 6-well plates fit together in the way described in this protocol and that the amount of 1 ml of medium used for the medium–air interface is adjusted only to the culture ware specified (24 mm Transwell® with a 0.4 µm Pore Polyester Membrane Insert Corning B.V., Cat. No. 3450 used together with Corning 3516 Costar 6 Well, Corning B.V., Cat. No. 3516). If you choose to use other culture ware, begin with 0.5 ml medium and add in 0.1 ml steps until the medium makes contact with the insert from the lower side. You need to save space, up to 20 hair follicles can be lined up on a single Transwell® insert instead of 10.
5. Alternatives for hypoxic conditions: Use hypoxic incubators, if available. If only incubators with normoxic conditions are available, cultivate the cells in hypoxic containers or in sealed

polyvinyl bags with a built-in valve, filled with hypoxic gas mixture. Preparation of a hypoxic nylon bag: Roughly, a 400 mm length of a 250 mm nylon sleeve is used to prepare a nylon container. Double-seal the sleeve with a heat-sealing device to ensure insulation. Prepare a 5 mm perforation by melting through the wall of a nylon bag with a heated metal screwdriver or size 10 wrench key. Mount a two-part screw-on plastic valve across the 5 mm opening. Place the cell flasks into a nylon bag, double-seal it from the other side and test the insulation. The bag should be air-tight. Release the valve and gently press the bag to empty the normoxic air out. If hypoxic gas mixture is available in pressured bottles, connect the bag valve to the bottle valve. If you are using a gas mixing device such as DASGIP MX4, set the gas mixture to 5 % O₂/5 % CO₂: Open the gas valves/switch on/all/mix/set gas % with a C (for CO₂) and O (for O₂) button until the right concentration appears on the display. Fit the plastic hose from the gas-mixing device onto the valve and fill the bag with hypoxic gas mix. Dismount the hose from the valve; press the bag gently to empty it again. Repeat (fill with hypoxic gas mix and empty the bag once more). Finally, fill with hypoxic mix to 80 % volume and close the valve, or compress the hose with a screw-on frame. The bag should remain slightly loose. Do not fill the bag tightly, as this will result in increased pressure once the gas expands at 37 °C in the incubator. Wipe the bag from the outside with a disinfecting tissue, let it dry for 30 s and place it in the incubator.

6. Documentation: Documenting the culture with photographs is advised with each medium change or at least once a week. You can select the time points that might be of importance for you. Choose a clearly visible, representative spot using a microscope equipped with a camera and photograph at 4× and 10× or 20× magnification. In case of a particularly important or distinctly representative event that you wish to record, use higher magnification. Do not lose track of time, since the HUMORS cultures should not be left out for too long for reasons of normoxia, room temperature, agitation, and possible contamination. Document at least three follicles per proband. Attempt to document the same spots with each inspection. This helps a great deal when evaluating the proliferation dynamics. Marking the lid of the 6-well plate helps to a certain extent, but you still might have to look for the spots again, since the Transwell® inserts can slightly rotate in the course of replacing the medium and the adherent cells still tend to alter their position in the culture. As the follicles are relatively easy to spot and recognize, this will not be a problem.

Keep track of the growing follicle ratio and count them every time you check. Quantify the surface of the growing follicles using the surface analysis function available with every photo software package [30].

7. Outgrowth culture: The cells that leave the ORS and populate the nylon mesh of the Transwell® insert will be a heterogeneous collection of stem cells, transiently amplifying stem cells, precursors and differentiated cells. Do not be puzzled by their various morphologies. At this stage, the two most important things are that the cells indeed leave the ORS and continue proliferating on the mesh and that they do not get contaminated. Typically, small, almost round cells will be present in this culture along with small bipolar cells and slightly larger bipolar spindle-like cells. Very large spindle-like cells are most likely fibroblasts and they are usually grouped around the proximal part of the ORS; they will proliferate far more quickly than the other cells and soon form the characteristic pattern recognizable for fibroblasts. Unfortunately, a culture that is plentifully generating fibroblasts at an early stage will most probably not yield much more than just that, since they will overgrow all the other cells.
8. Colonies: Cells proliferating on the mesh will begin to form aggregations (colonies) after roughly a week of medium–air interface cultivation. This feature is not a guarantee of their stem cell-like nature, but it is a good sign. Colonies are not a prerequisite for a successful cultivation. Rather, two-dimensionally proliferating cells that form a confluent spreading layer around the follicle are a better material for the adherent culture.
9. Individual differences: The cultivation times involved to the point of flooding, harvesting, transfer to adherent culture and characterizing may differ between donors, depending on the proliferation rate of their cells. Common for all the probands: The time point of harvest and start of the adherent culture is tagged as zero passage (p0) and the start of subculturing as the first passage (p1).
10. Adherent culture of the ORS cell pool: Your starting adherent culture (p0) will be as heterogeneous as the outgrowth culture described in detail in **Note 6**. There is no reason to worry, since you will have a chance to selectively facilitate melanocytes and get rid of other cell types in the course of further cultivation.

HUMORS adherent culture: As you change the medium, leave a small amount (less than 10 %) of conditioned medium in the cell vessel. The conditioned medium appears to contain secreted paracrine growth factors. Some will call this sheer superstition, but it works. Up to the point of advanced selected adherent culture, the cells could be in a stage of melanoblasts,

amelanotic or melanotic melanocytes. The expression of melanocyte markers begins to be apparent after p3 and the highest expression has been registered between p8 and p12. After p15, the HUMORS may become unstable in melanin production. To be on the safe side, use the cells by p16.

11. Selection of melanocytes: Geneticin challenge and differential trypsinization. As a rule, combining a single Geneticin treatment with regular 4 min differential trypsinization results in a pure culture of melanocytes that is easy to maintain (Fig. 3).

Suboptimal conditions of trypsin digestion (room temperature and shorter time) limit proteolytic activity and efficiency in detaching cells. The result of such a procedure is the detachment of those cells that adhere more weakly than the others. The very first fraction to detach will be melanocytes and they can be collected for further passaging. Of course, the sensitivity of this method is not high, therefore you are bound to lose a certain portion of melanocytes that still remain adherent at the 4-min point. Moreover, some of the other cells of the primary culture, keratinocytes and fibroblasts, that may detach more quickly, will end up in your 4-min-fraction. The percentage of these will decrease with routine passaging and should remain within a couple of percent at most. Regular differential trypsinization will maintain the purity of your melanocyte culture with constant efficiency. If you find fibroblasts or keratinocytes in your melanocyte culture again, reduce the time of differential trypsinization to 3.5 min, and if necessary further down to 3 min. If the fibroblasts and/or keratinocytes persist, repeat Geneticin treatment.

Continue differential trypsinization or not? As a rule—yes. It is advisable to use differential trypsinization at every passage. In a clear case, where all HUMORS look absolutely right and no pebble-like or spindle-like morphologies can be observed, use complete trypsinization. This is justified if you are engaging the cells in an experiment that does not involve many further passages, since it will save you some melanocytes that would still remain adherent after 4 min. If the morphologies differ, you should continue with differential trypsinization.

12. Expression profile of HUMORS: HUMORS will express melanocyte-characteristic genes in much higher levels than human embryonic stem cells or any other pluripotent control. Expect residual expression of pluripotency genes in early passages of the HUMORS adherent culture; these very low levels of expression occur due to incomplete differentiation stages of HUMORS and hint towards their stem cell origin. For the same reason, their expression levels of melanocytic genes may be lower than that of NHEM. End-stage melanotic HUMORS will not express pluripotency genes.

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Pancreas Development Ex Vivo: Culturing Embryonic Pancreas Explants on Permeable Culture Inserts, with Fibronectin-Coated Glass Microwells, or Embedded in Three-Dimensional Matrigel™

Hung Ping Shih and Maike Sander

Abstract

Pancreas development is a complex and dynamic process orchestrated by cellular and molecular events, including morphogenesis and cell differentiation. As a result of recent explorations into possible cell-therapy-based treatments for diabetes, researchers have made significant progress in deciphering the developmental program of pancreas formation. In vitro pancreas organ culture systems provide a valuable tool for exploring the mechanisms of gene regulation, cellular behaviors, and cell differentiation. In this chapter, we review three common techniques for culturing embryonic pancreas explants. Each technique is suitable for different applications. Specifically, culturing embryonic pancreas on culture inserts provides an excellent platform to test the effects of chemical compounds. Conversely, when the embryonic pancreas is cultured in fibronectin-coated glass microwells, the system provides unique culture conditions to monitor organ growth and cellular dynamic events. Lastly, when the embryonic pancreas is embedded in Matrigel, organogenesis can be studied in a three-dimensional environment instead of limiting the analysis to one plane.

Key words Pancreas, Development, Explants, Differentiation, Morphogenesis

1 Introduction

The pancreas is an essential organ for nutrient metabolism, with both endocrine and exocrine features. The main function of the endocrine pancreas is to regulate blood glucose homeostasis; the exocrine pancreas, conversely, is primarily digestive. Malformation of either the exocrine or the endocrine cells, or both, leads to diseases such as diabetes, pancreatitis, and pancreatic cancer.

Both exocrine and endocrine cells arise from a common pancreatic progenitor cell pool during pancreas development. In mice, pancreas organogenesis starts at embryonic day (E) 8.5–E9.0, when the first epithelial endoderm-derived pancreatic progenitor cells invaginate into surrounding mesenchymal tissues. Over the next 3 days,

pancreatic progenitor cells proliferate and the majority of the progenitor cells remain pluripotent. During these early stages, the pancreas expands significantly in size, but overall morphology initially undergoes little change [1]. By E12.5, pancreas morphogenesis is initiated and cell differentiation begins [1, 2]. From this stage onwards, pancreas development is marked by the development of a separate endocrine and exocrine compartment. At birth, cell differentiation is completed and the pancreas has assumed the morphology and cytoarchitecture of the mature organ.

The last two decades have provided significant insight into the molecular mechanisms controlling pancreatic cell lineage choices and cell differentiation. Recent investigations have also explored how pancreas organogenesis is coordinated with endocrine and exocrine cell differentiation (for review, *see* refs. 3, 4). Our knowledge of pancreas development is mainly based on *in vivo* genetic studies using animal models (primarily transgenic mouse models). However, there are significant limitations to generating and analyzing transgenic mouse models—specifically, the inherently slow nature of genetic research in genetic mouse models. Moreover, due to the highly dynamic nature of pancreas morphogenesis, studies using end-stage analysis often fail to provide sufficient information to understand the cellular events that occur throughout the developmental process.

To overcome these difficulties, numerous studies have established different culture conditions and strategies for studying pancreas development *ex vivo* [5, 6]. These techniques now allow the monitoring of pancreas morphological changes and cell differentiation in real time. *Ex vivo* culture systems also allow for testing the effects of compounds on pancreatic development.

2 Materials

All reagents are to be prepared under a cell culture hood; only cell culture grade reagents are to be used. All hardware and reagents are to be handled with standard sterile techniques.

2.1 Cell Culture Supplies

1. Glass-bottom culture dish:
 - Dish diameter: 35 mm.
 - Coating: Uncoated.
 - Glass thickness: No. 0–1.5.
 - Cover slip (choose a thickness appropriate to the lens used, *see* **Note 1**): Glass diameter: 10 mm.
2. 4-Well × 1 mL MultiDish cell culture dish with round wells and lid, cell-culture-treated polystyrene, sterile.
3. Nuclepore Track-Etched Membranes (13 mm, 1.0 μ m pore).
4. Tissue culture dishes (10 mm).

2.2 Reagents

1. DPBS (Dulbecco's phosphate-buffered saline).
2. Pancreas culture medium.
The culture medium containing DMEM/F12 medium with HEPES, L-glutamine, and without phenol red, supplemented with 10 % FBS (fetal bovine serum), and antibiotic/antimycotic agents (100 U/mL penicillin, 100 µg/mL streptomycin) (*see Note 2*).
3. Purified human serum fibronectin (hFN).
Prepare stock hFN solution (1 mg/mL) by reconstituting 5 mg lyophilized hFN1 in 5 mL sterile pure water. Let the solution sit on ice for 5 min; do not agitate or swirl it, as this will cause the fibronectin to precipitate out of solution. Prepare aliquots of 100 µL each, and store at -80 °C. After thawing, keep the hFN solution at 4 °C for no more than 2 weeks.
4. Matrigel™.
BD growth factor-reduced (GFR), phenol red-free Matrigel™.
Thaw BD Matrigel™ Matrix GFR phenol red free by submerging the vial in ice in a 4 °C refrigerator overnight. Swirl vial to ensure that Matrigel™ is evenly dispersed. Keep Matrigel™ on ice at all times to prevent solidification.

2.3 Preparation of hFN-Coated Glass-Bottomed Culture Dish

1. Thaw the hFN stock solution (1 mg/mL) slowly on ice. Do not agitate or swirl it. If the fibronectin has aggregated into insoluble clumps or transparent filaments, discard the aliquot and use a new one.
2. Further dilute stock hFN solution to 0.1 mg/mL with sterile pure water.
3. For each glass-bottomed dish (with 10 mm glass slips), add 100 µl 0.1 mg/mL hFN solution onto the glass slips. Make sure that the solution covers entire surface of the glass slips.
4. Let the hFN solution dry completely at room temperature in the cell culture hood; allow 2 h for drying. After drying, the hFN-coated plates can be stored at 4 °C for no more than 4 weeks.

3 Methods

3.1 Mouse Embryos, Pancreas Dissection, and Tissue Preparation

Chill sterile DPBS and DMEM/F12 medium on ice. Sterilize dissecting area by spraying with 70 % ethanol.

1. E0.5 is defined as midday on the day of emergence of the vaginal plug. By E11.5 (*see Note 3*), sacrifice pregnant dams with CO₂ inhalation, followed by cervical dislocation. The uteri are subsequently isolated.
2. Extract the string of embryos, and place intact in a 10-cm petri dish containing ice-cold DPBS.

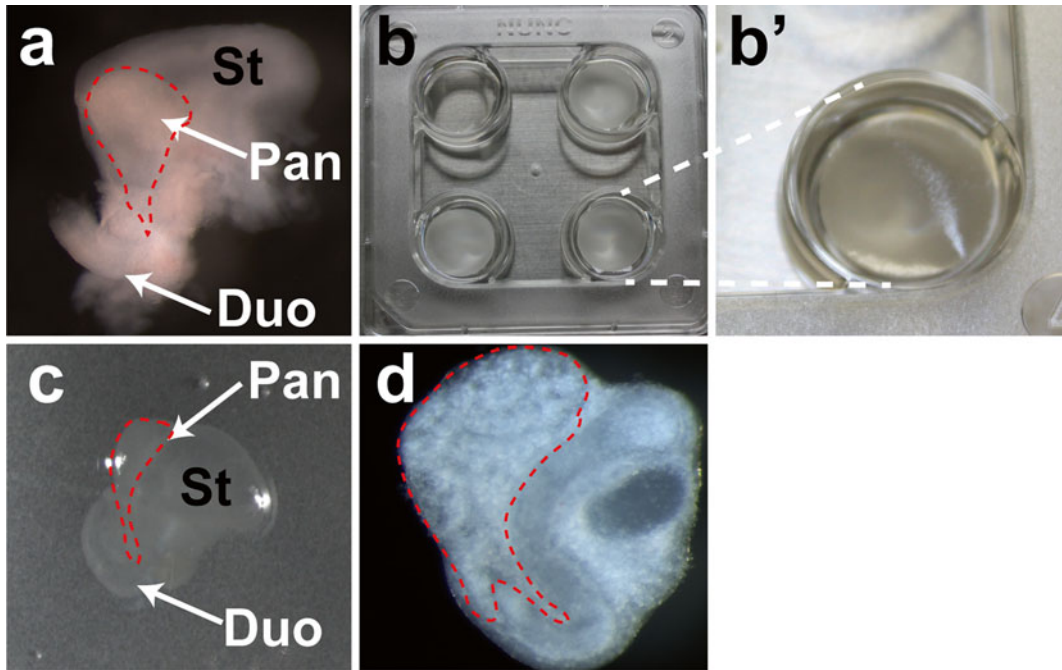


Fig. 1 Illustration of pancreas explants on cell culture inserts. **(a)** Example of a dissected E11.5 pancreas. The stomach and duodenum are still attached to the pancreas. Note the *red dashes* outlining the dorsal pancreas for illustrative purposes. **(b)** Nuclepore Membrane inserts floating on top of the liquid medium. For comparison purposes, the well in the *upper left corner* has no insert. **(b')** Zoomed-in image of *right lower corner*, showing that filter is floating. Note membrane orientation (*reflective side up*). **(c)** Explant tissue, shown growing at air/medium interface. Stomach and duodenum are still attached to pancreas, for illustrative purposes. **(d)** After 3-day culturing, the dissected E11.5 pancreas explants expand, and develop branching structures. *St* stomach, *Pan* pancreas, *Duo* duodenum. (Color figure online)

3. Dissect each embryo by opening its abdominal wall using fine forceps under a dissecting stereomicroscope. The stomach and liver are identified first; the dorsal pancreas is located on the left ventral side of the stomach (Fig. 1a).
4. Separate the stomach and liver tissues from the embryonic pancreas and duodenum, which are left attached to each other; transfer the embryonic pancreas and duodenum into ice-cold DMEM/F12 medium using a pre-rinsed pipetman tip (*see Note 4*). The dissected tissue can be stored in this condition for no more than 4 h before culturing.

All three-culture techniques described herein use the same tissue preparation procedure.

3.2 Pancreas Explants on Cell Culture Inserts

This method is particularly useful for studying the effects of chemical compounds on pancreatic development. Pancreatic buds can be cultured in this condition to up to 7 days, thus allowing quantitative exploration of cell proliferation and gene expression. Due to the potential for inconsistencies in experimental conditions, it is

advisable to incorporate multiple experimental replicates, so as to allow statistical analysis and reproducible results.

1. Pre-warm explant medium; allow 1 h in 37 °C water bath.
2. Add 1 mL pre-warmed explant medium into each well of the MultiDish cell-culture dish. Keep the liquid surface level; avoid introducing air bubbles.
3. Using forceps, extract one Nuclepore Track-Etched Membrane from separator paper inserts (*see* **Note 5**); identify the membrane orientation and find the reflective side (Fig. 1b, b').
4. Keeping the reflective side up, place the membrane on the surface of the explant medium without disrupting the surface tension. If the membrane sinks into the medium, remove it with forceps, dispose of it, and then repeat the procedure with a new membrane.
5. Use a 20- μ L pipette with a pre-rinsed pipetman tip to transfer the pancreatic tissue from its storage in ice-cold DMEM/F12, to the top of the Nuclepore Membrane. The tissue should grow at the air/medium interface (Fig. 1c). During the transfer, avoid introducing air bubbles or excessive medium on the surface of the membrane.
6. Add 20 μ L of explant medium onto the air/medium interface, and reorient the tissue. The dorsal pancreas must be ventral side up, and the tissue must not be folded. This is critical to ensure consistency of tissue morphology at the end of the culture period. Place the cultures in a tissue-culture incubator (37 °C; 5 % CO₂).
7. The pancreatic tissue will flatten out in the culture medium; allow an hour for this to occur. After 24 h, the branches of the pancreas will have developed enough to be visually identifiable (Fig. 1c, d).

Change the explant medium daily; at each change, add 20 μ L of fresh pre-warmed medium to the air/medium interface to reduce contamination.

3.3 Pancreas Explants on hFN-Coated Glass-Bottomed Dishes

This method, when combined with real-time microscopy and fluorescence labeling, is particularly useful for the study of pancreatic branching morphogenesis and behavior of individual cells [6, 7]. Many recent studies have used genetic strategies to label different cell types with either membrane-associated or nuclear fluorescent proteins, thereby allowing the tracking of individual cell behaviors. Single-cell imaging techniques, using four-dimensional live imaging, have been established for the early developmental stages of both the pancreas and kidney [6, 8]. Because the hFN substrate causes the pancreatic tissue to flatten over time and thereby become thinner (Fig. 2a, b), the tissue poses less of an obstruction to the light from the microscope, which facilitates imaging.

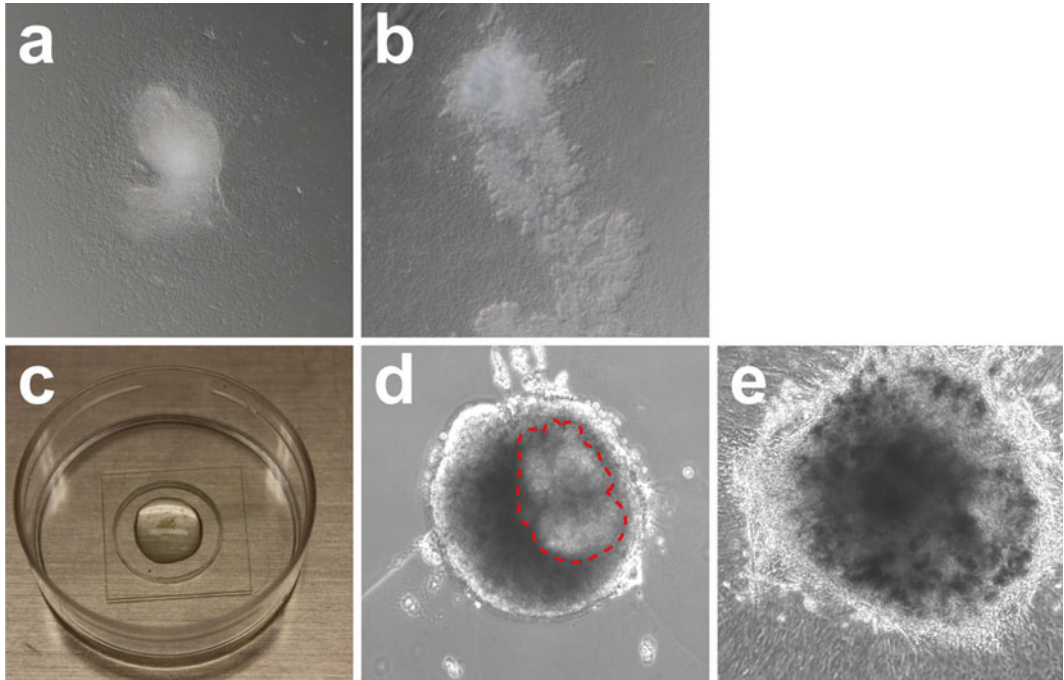


Fig. 2 Pancreas explants on hFN-coated glass-bottomed dish and embedded in three-dimensional Matrigel™. **(a)** E11.5 pancreas explants grow on hFN-coated glass-bottomed dish for 24 h. Note that the tissue has attached to the glass bottom and flattened out at the edges. **(b)** After 5 days in culture, pancreas explants further spread out and develop branching structures. **(c)** E11.5 pancreas explants embedded in solidified Matrigel™ on glass microwells of MatTek™ dishes. **(d)** After 24-h culturing, the dissected E11.5 pancreas explants retain round morphology. Note that the pancreas epithelium can be identified as outlined with *red dash lines*. At this stage, the explants start to undergo morphological changes. **(e)** After 3 days in culture, the edge of the explants spreads out while the whole tissue remains encapsulated in the Matrigel™. The branched tip structures can be readily identified

1. Pre-warm explant medium in 37 °C water bath; allow 1 h for warming.
2. Add 200 µL of pre-warmed explant medium directly into the hFN-coated glass slips of MatTek™ dishes (*see Note 6*). Incubate at room temperature for 20 min and then aspirate the medium. Add 100 µL of fresh pre-warmed explant medium into the hFN-coated glass microwells of MatTek™ dishes.
3. Using a 20-µL pipette with a pre-rinsed pipetman tip, transfer the pancreas explants onto the hFN-coated glass microwells so that the tissue can settle to the bottom.
4. Reorient the tissue by gently pipetting the medium; avoid introducing air bubbles. The dorsal pancreas must be ventral side up, and the tissue must not be folded.
5. Slowly aspirate the medium using a 200-µL pipetman until the explant tissue flattens out in the glass microwells. Add 10 µL of fresh explant medium onto the glass to keep the tissue wet.

6. In order to keep the tissue moisturized inside the dishes, slowly add 1 mL explant medium to the edge of the MatTek™ dishes; ensure that no medium overflows into the microwells.
7. Place the cultures in a tissue-culture incubator (37 °C; 5 % CO₂); allow 45 min for the explants to attach to the glass-bottomed microwells.
8. To verify whether the tissue has properly attached to the glass-bottomed microwells, slowly add 1 mL of explant medium into the dish, ensuring that the medium covers the entire surface. If the explants detach from the glass-bottomed microwells, the procedure has failed; aspirate all medium and start again from **step 2** of Subheading 3.3.
9. Place the cultures in a tissue-culture incubator (37 °C; 5 % CO₂) and change the explant medium daily.

3.4 Pancreas Explants Embedded in Three-Dimensional Matrigel™

This method is particularly useful for the three-dimensional study of cell/extracellular matrix (ECM) interactions. In this section we demonstrate the use of Matrigel™, a proprietary combination of (primarily) laminin and collagen. Recent studies have successfully used hydrogel supplements with different assortments of ECM molecules to generate culture environments in which both pancreatic progenitor cells [9] and adult pancreatic ductal progenitor cells [10] can be maintained. When combining with live-imaging techniques, this method can also serve as a platform for investigating branching morphogenesis in 3-D [11].

1. Thaw Matrigel™ as previously explained. Chill DMEM/F12 medium, Eppendorf tubes, and pipetman tips on ice. Pre-warm explant medium in 37 °C water bath; allow 1 h for warming. Use only ice-chilled pipetman tips and Eppendorf tubes to handle Matrigel™.
2. Transfer pancreas explants with 100 µL ice-cold DMEM/F12 into an ice-chilled Eppendorf tube. Pipette and mix 100 µL Matrigel™ with the explant with medium (50 % dilution). Avoid introducing excessive air bubbles in this step.
3. Place the Matrigel™/explant mix on ice so that air bubbles can escape; allow 10 min for this step.
4. Transfer 200 µL of Matrigel™/explant mix into uncoated glass microwells of MatTek™ dishes. Avoid introducing air bubbles and overflow.
5. Place the cultures in a tissue-culture incubator (37 °C; 5 % CO₂) so that the Matrigel™ will solidify completely; allow 1 h (Fig. 2c).
6. Slowly add 2 mL of the explant medium into the MatTek™ dishes from the side. Ensure that the solidified Matrigel™ is not disrupted and that the medium covers the entire surface.
7. Place the cultures in a tissue-culture incubator (37 °C; 5 % CO₂) and change the explant medium daily (Fig. 2d, e).

4 Notes

1. Coverslip number and thickness index:

Coverslip No.	Thickness (mm)
0	0.085–0.13
1.0	0.13–0.16
1.5	0.16–0.19
2.0	0.19–0.23

- For explants with fluorescent tags, we recommend the use of the F12/DMEM medium without phenol red, to avoid quenching effect.
- In this chapter we only show procedures using E11.5 embryos. Earlier or later stages of embryonic pancreatic development can also be successfully cultured using the same conditions. We have tested all three culture procedures using embryonic pancreas dissected from E9.5 to E15.5 with success. However, pancreases of earlier stages than E11.5 are more difficult to orient properly, and the end results may show greater variation.
- Pre-rinse the 200- μ L pipetman tip with explant medium to lubricate the tip wall and prevent tissue sticking to the tip. If the tissue size is larger than the opening, cut the opening to enlarge the holes.
- Here we used Nuclepore Track-Etched Membranes (diameter: 13 mm; pore size: 1 μ m). This is the only pore size recommended: larger pore sizes may lead to the membrane sinking below the surface of the liquid, and smaller pore sizes may compromise liquid exchange efficiency and cultured tissue morphology.
- To avoid overflow, add no more than 200 μ l of medium. This step is to wash off excessive salt and impurities deposited on glass slips during hFN coating procedure.

Acknowledgements

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Ultra-rapid Manufacturing of Engineered Epicardial Substitute to Regenerate Cardiac Tissue Following Acute Ischemic Injury

Vahid Serpooshan and Pilar Ruiz-Lozano

Abstract

Considering the impaired regenerative capacity of adult mammalian heart tissue, cardiovascular tissue engineering aims to create functional substitutes that can restore the structure and function of the damaged cardiac tissue. The success of cardiac regenerative therapies has been limited mainly due to poor control on the structure and properties of the tissue substitute, lack of vascularization, and immunogenicity. In this study we introduce a new approach to rapidly engineer dense biomimetic scaffolds consisting of type I collagen, to protect the heart against severe ischemic injury. Scaffold biomechanical properties are adjusted to mimic embryonic epicardium which is shown to be optimal to support cardiomyocyte contractile work. Moreover, the designed patch can serve as a delivery device for targeted, controlled release of cells or therapeutic macromolecules into the lesion area.

Key words Cardiac tissue engineering, Myocardium regeneration, Embryonic epicardium, Scaffold, Type I collagen, Cardiomyocyte, Angiogenesis

1 Introduction

Engineering of three-dimensional (3D) cardiac tissue substitutes, to date, has presented new opportunities for developing alternative cardiac regenerative therapies, as well as biomimetic in vitro models for basic cardiovascular research [1–6]. Different approaches that have been used to generate cardiac tissue constructs include (a) formation of 3D cardiac cell aggregates (embryoid bodies) [7–10]; (b) employing naturally occurring, decellularized tissues which are used as scaffolds [11–13]; and (c) utilizing either natural or synthetic scaffolding biomaterials (proteins) such as collagen, gelatin, and alginate as a substratum for tissue reconstruction [4, 5, 14]. Cell aggregates (a) and naturally derived matrices (b), however, have shown limited success primarily due to the poor control on the ultrastructure and physical-mechanical properties of the scaffold, insufficient vascularization, and risk of thrombosis [15–18].

In vitro-reconstituted, type I collagen scaffolds have attracted growing interest as biocompatible biomaterials for use in a wide variety of biomedical applications, particularly, in tissue regeneration, through providing tissue-like milieu for cell growth and integration [19–22]. Main advantages of these hydrogels include facilitated mass transfer and cell migration within the construct, which are favored by the scaffold fibrillar structure, biocompatibility, and biodegradability [17, 19, 20].

This methods chapter introduces a new technology to produce dense collagen-based scaffolds, endowed with optimized biomechanical properties to be used: (a) as a 3D in vitro model to study cardiac cell-extracellular matrix interactions [21–23], and (b) as a cardiac patch to aid regeneration of cardiac tissue in vivo [4, 5, 17]. The latter function of the patch can be conducted in multiple ways including (1) providing a temporary mechanical support for the damaged tissue and retarding adverse remodeling processes, (2) offering a biomimetic microenvironment allowing for cell migration and neovascularization, (3) promoting cardiogenesis of resident cardiac stem cells, and (4) providing controlled, targeted delivery of cells or drugs into the lesion area [4, 5]. Plastic compression of highly hydrated collagen gels is used to rapidly produce dense tissue scaffolds with elastic moduli approaching to that of embryonic epicardium, which is shown to be optimal to support cardiac cell contractility [24–28].

2 Materials

Prepare and store all reagents at room temperature (unless indicated otherwise). Carefully follow waste disposal guidelines and regulations when disposing waste materials.

2.1 Collagen Gel Preparation

1. 1× high-glucose Dulbecco's modified Eagle medium (DMEM).
2. Rat tail-derived type I collagen solution in acetic acid (3.84 mg/ml).
3. 1× phosphate-buffered saline (PBS).
4. NaOH solution (0.1 M, in sterile PBS).
5. Ice bucket.
6. Tissue culture 24-well plates (15.6 mm in diameter) are used as mold to cast collagen into (*see Note 4*).
7. Absorbent paper blot layers, a steel mesh, two polymer meshes, and four fabric meshes (Fig. 1).

2.2 Mechanical Characterization of the Patch

1. Atomic force microscopy (AFM) custom-designed flat tips (Fig. 2). The tip is fabricated using focused ion beam milling and utilized to probe the gel stiffness.

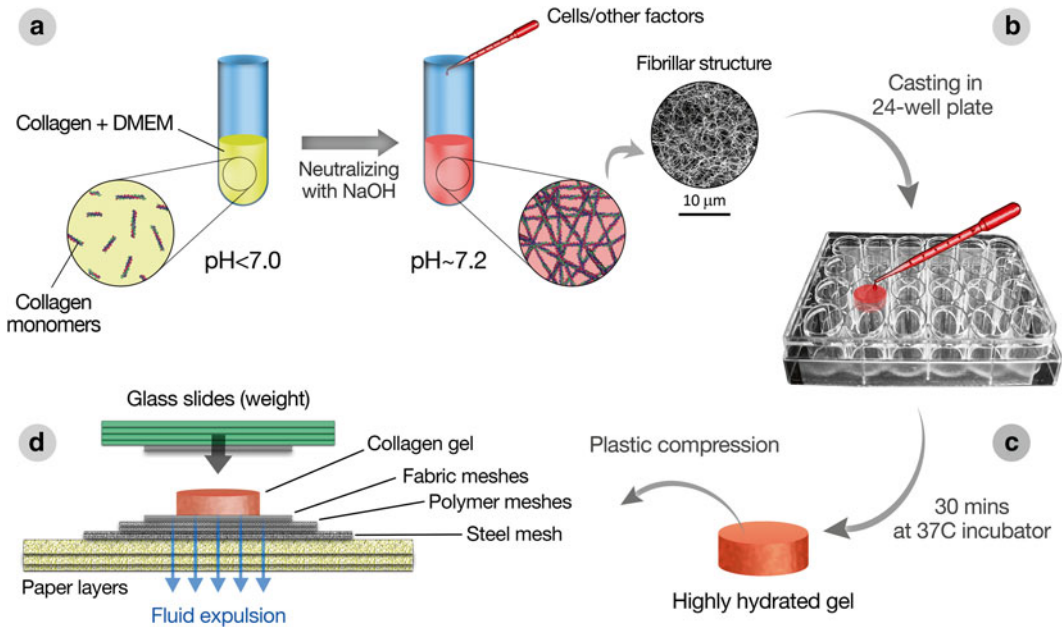


Fig. 1 Schematic demonstration of the experimental procedures to prepare cardiac patch. **(a)** Preparation of collagen solution, pre-neutralization (*left*) when collagen monomers are free floating (acidic liquid phase), and after adding NaOH (*right*) which initiates polymerization of collagen monomers and formation of a 3D fibrillar network. Cells and other factors will be added right after adding NaOH. **(b)** Casting the neutralized collagen solution into the wells of 24-well plate. **(c)** Incubation of cast gels in the incubator for 30 min at 37 °C. **(d)** Experimental setup used to apply plastic compression to the highly hydrated collagen gel (*see text for details*)

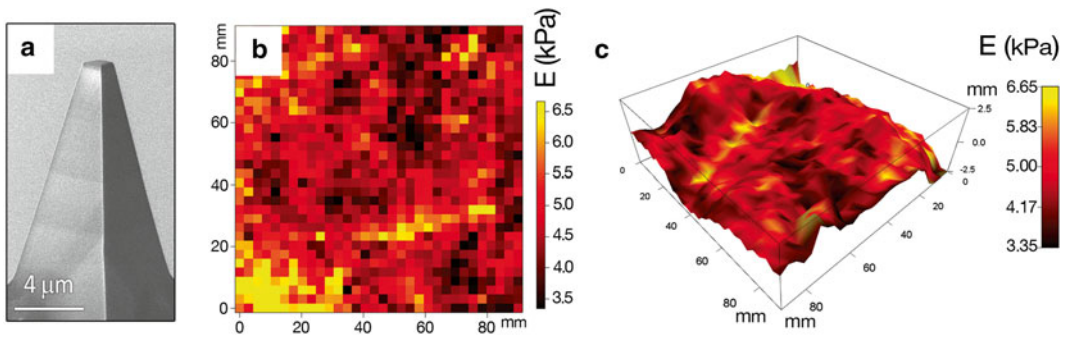


Fig. 2 **(a)** The custom-made flat tip to be used in atomic force microscopy (AFM) of the patch, manufactured using electron beam deposition and utilized to probe the stiffness of the gels in scanning areas of 90 × 90 μm **(b)** and **(c)**

2.3 Mouse Model of Ischemic Heart Injury: Surgical Procedure of Patch Grafting

1. Male 10–13-week-old C57BL/6J mice (purchased from Jackson Laboratories, Bar Harbor, ME, USA).
2. Isoflurane (100 mg/kg, inhalation) and isoflurane inhalational chamber (for mice).
3. 22-G angiocatheter.

4. Small animal volume-control ventilator.
5. 7-0 suture which is used to ligate the artery, hence, inducing infarct.
6. 8-0 suture which is used to attach the patch onto the ischemic tissue.
7. Hair removal (shaving) cream.

2.4 Histology and Immuno-histochemistry

Histological analysis was conducted following standard protocols for paraffin embedding.

1. 10% neutral buffered formalin solution: Reagents: Formaldehyde (37–40 %, 100 ml/l), distilled or deionized water (900 ml/l), sodium phosphate, monobasic (4.0 g/l), and sodium phosphate, dibasic (6.5 g/l).
2. Balanced sterile saline solution.
3. Hematoxylin and eosin (HE) staining reagents.
4. Masson's trichrome (TC) three-color staining reagents: Weigert's iron hematoxylin, and then three different solutions labeled A, B, and C.
5. von Willebrand factor (vWF) antibody (staining reagent) (Sino Biological, Beijing, China).
6. Antibodies for immunohistochemical analysis:
 - Cardiomyocytes: α -Actinin (Sigma A7811, MO, USA).
 - Cardiac fibroblasts: Discoidin domain receptor 2.
 - T-Cells: Cluster of differentiation 3 (CD3, Abcam ab6326, MA, USA).
 - Epicardial derived progenitor cells: Platelet-derived growth factor receptor α (PDGFR α , Abcam ab61219, MA, USA).
 - Smooth muscle cells: Alpha smooth muscle actin (α -SMA, Sigma A5228, MO, USA).
 - Macrophages: F4/80 (Abcam ab100790, MA, USA).

3 Methods

Perform all procedures at room temperature (unless otherwise specified). The procedure described below is for preparing two gels/run.

3.1 Collagen Gel Preparation

1. Add 0.5 ml of 1 $\times$ DMEM to 0.9 ml of sterile type I collagen solution (3.84 mg/ml) in a 5 ml tube (*see Note 1*).
2. Neutralize the mixture using ~48 μ l of 0.1 M NaOH (*see Note 1*).
3. Rapidly add 0.6 ml of 1 $\times$ DMEM to the neutralized solution. The added media at this step can contain cells and/or factors

that are going to be seeded within the patch. Since the mixture is still in liquid phase (kept on ice), addition of factors/cells at this stage will assure their 3D uniform distribution within the gel structure (*see* **Notes 2 and 3**).

4. Cast the 0.9 ml of prepared solution (2×) into each well of a 24-well tissue culture plate and gently place it in a tissue culture incubator for 30 min at 37 °C for polymerization (*see* **Note 4**).

3.2 Plastic Compression of Cast Hydrated Gels

1. Gently transfer the cast highly hydrated gels from the incubator to a tissue culture hood (when the patch needs to be sterile, otherwise use the bench). Using a scalpel smoothly detach the cast gels from the wells of 24-well plate. Cut through the periphery of the gel and make sure that the entire patch is detached from the wall.
2. Place a large glass slide (51×75×1 mm³) on top of the well where the gel is removed and flip the plate. Apply a slight shake if needed to transfer the gel onto the glass slide. Slide the gel onto a porous support comprising (bottom to top) several layers of absorbent paper blot layers, a steel mesh, two polymer meshes, and two fabric meshes (Fig. 1) (*see* **Note 5**).
3. Place two fabric meshes on top of the gel located on porous substrate (Fig. 1).
4. Apply a static compressive stress of desired magnitude to the highly hydrated scaffold, via placing a stack of glass microscope slides (75×25×1 mm³) on top of the fabric meshes (Fig. 1). Apply the force for 2–5 min in order to remove excess water within the gel and produce a dense biomaterial with improved biological and mechanical properties [21, 24, 28, 29]. In case of preparation of *epicardial patch*, apply 1,400 N/m² for 5 min [4]. Considering the surface area of each gel to be equal to a well size in 24-well plate (1.91 cm²), this compressive stress is equal to the load of 27.3 g of glass slides on top of the gel (*see* **Note 6**).

3.3 Atomic Force Microscopy: Measurement of Patch Elastic Moduli

1. Using AFM in nanoindentation mode, with indentations of ~100 nm (to minimize the influence of substrate-related artifacts), elastic moduli of the compressed collagen gels can be measured by scanning areas of 90×90 μm (Fig. 2) (*see* **Notes 7 and 8**).

3.4 Mouse Model of Myocardial Infarction (MI): Epicardial Grafting of the Patch

Male 10–13-week-old C57BL/6 J mice are studied in different experimental groups as below (*see* **Note 9**):

- A. Control mice which undergo sham ligation with no infarct/patch treatment.
- B. MI-only group which have infarct with no treatment.

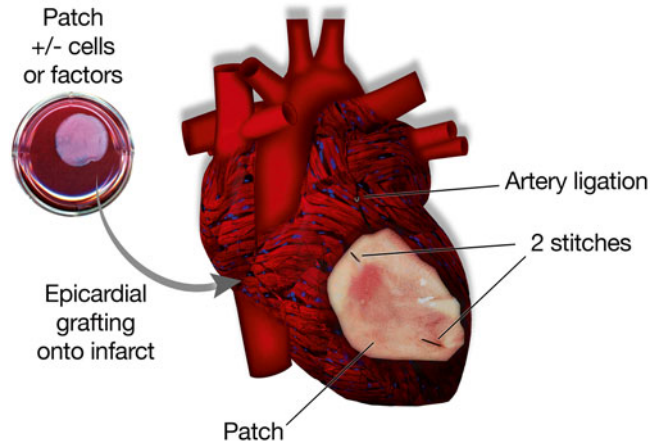


Fig. 3 Schematic demonstration of the surgical procedure to induce myocardial infarction in the mouse heart, followed immediately by patch implantation

- C. Healthy mice (with no infarct) treated with the patch.
 - D. Infarcted myocardium grafted with patch alone.
 - E. Infarcted myocardium grafted with patch laden with cells/factors.
1. Anesthetize the mice using an isoflurane inhalational chamber (100 mg/kg, inhalation), shave their chest, and place them on a heated platform in the left lateral decubitus position (to facilitate echocardiography).
 2. In vivo heart function of all animal groups must be recorded pre-surgery as baseline. Two-dimensional (2D) analysis is performed using an ultrasound platform. 2D clips and M-mode images are obtained using a short-axis view from the mid left ventricle (LV) at the tips of the papillary muscles (*see Notes 10 and 11*).
 3. After pre-surgery echo, mice will be anesthetized again, followed by endotracheal intubation using a 22-G angiocatheter that is connected to a small animal volume-control ventilator.
 4. Perform a left thoracotomy via the fourth intercostal space. Retract the lungs to expose the heart. Tear off the pericardium.
 5. Using a 7-0 suture, tightly ligate the left anterior descending (LAD) artery approximately 2–3 mm below the edge of the left atrium (Fig. 3) (*see Note 12*).
 6. For experimental groups treated with patch: Gently place the prepared collagen patch onto the ischemic area (with color change), stretch it to cover the whole damage area (and area at risk), and then using two 7-0 sutures (Fig. 3) secure the patch onto the surface of the ischemic myocardium (*see Note 13*).
 7. Once the patch is grafted, close up the chest and suture the muscle and skin tissues. Animals shall be kept on a heating pad until they recover.

8. At weeks 2 and 4 postsurgery (patch implantation) repeat the echo (**steps 1** and **2**) to monitor the patch effect of cardiac function in vivo.
9. Animals are usually sacrificed after the echo at week 4. Follow the standard regulations set by the animal care authorities to euthanize the animals.
10. Dissect the heart (with/without patch) quite gently for histologic analysis (*see* **Note 14**).

3.5 Histology and Immunohistochemistry Analysis

Histological analysis shall be performed following standard protocols for paraffin embedding.

1. Rinse the dissected hearts thoroughly with saline (BD Biosciences), dry them, and put them in Eppendorf tubes filled with fixative solution (10 % formalin). Keep the samples in fixative for 24–48 h.
2. Follow Stains file, a comprehensive online resource for histological technique [30], or Dako processing chapter by John Kiernan [31] for detailed information on standard procedure of paraffin embedding for four-chamber view of heart samples.
3. Mounted heart samples are stained for:
 - HE: to visualize nuclei in blue and cytoplasm and connective tissue in pink.
 - Masson's trichrome (TC): to label muscle fibers in red, collagen in blue, cytoplasm in pink, and nuclei in dark brown.
 - von Willebrand factor (vWF): to stain endothelial cells in the vessel walls in dark brown.
4. For immunohistochemistry, embedded samples are sectioned at a thickness of 7 μm . Antibodies to be used are listed in Subheading 2.4. Follow standard protocols for sample preparation for immunohistochemical analysis (e.g., Dako general instructions for immunohistochemical staining [32]) (*see* **Note 15**).

4 Notes

1. For the entire time of gel preparation, all the solutions must be kept on ice. Keeping collagen solution in cool temperature will avoid premature polymerization of collagen and will assure producing a homogenous gel.
2. In the case of adding cells to neutralized media, temperature sensitivity of the cells must be taken into account. The solution temperature (i.e., resting time on the ice) can be adjusted based on how sensitive/fragile the cells are.

3. When calculating the amount of cells/factors to be added to the collagen solution tube, keep in mind that the prepared solution in each tube is for *two* collagen gels.
4. **Steps 3 and 4** in Subheading [3.1](#) must be done as rapidly as possible, considering the fact that collagen gelation starts right after adding the NaOH (chilling the tube on ice will slow down gelation). In case collagen gelation initiates before casting the solution into the wells, the final gel will not set properly (will stay liquid).
5. Highly hydrated collagen gels undergo a gravity-driven consolidation (known as “self-compression” [[21](#), [25](#)]), under their own weight. Therefore, transfer the gel to porous substrate as quickly as possible.
6. A list of varying levels of plastic compression applied to hydrated collagen gels and resulting weight loss, collagen fibrillar density, and solid volume fraction are presented in Serpooshan et al. [[21](#)].
7. Custom-designed flat AFM tips need to be fabricated (e.g., via focused ion beam milling technique) and utilized to probe the stiffness of the soft gel (Fig. [2](#)).
8. Phase-contrast imaging and surface topography of the compressed collagens can also be performed using AFM, in semi-contact mode with rounded cantilevers ($R_{tip} \sim 40$ nm).
9. The entire procedures involving animal housing and surgeries must follow the rules and guidelines defined by the animal care authorities in each institute (e.g., Institutional Animal Care and Use Committee) and obtain their approval.
10. Echo parameters to be measured from LV dimensions in the 2D short-axis view include fractional shortening (FS, %), ejection fraction (EF, %, estimated by extrapolation of 2D data), LV internal diameter (LVID), and posterior wall thickness (LVPW), both at end diastole and systole.
11. It is quite important that echo measurements and analysis are performed by two independent observers blinded to study groups.
12. One exclusion criterion for the success if LAD ligation is to check for an immediate color change in the LV wall (red to pale).
13. Keep the patch sterile and hydrated, immersed in PBS or culture media until the time of implantation.
14. Pay extra attention at the time of heart dissection for not disturbing the heart-patch interface.
15. Another exclusion criterion for the in vivo patch engraftment study is to check that the patch has covered >70 % of the infarct area (controlled by histology).

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INDEX

A

Angiogenesis 152, 153, 163, 168, 170
Astrogliosis 11, 17

B

Biopsy 154, 155, 157, 169

C

Cell

bone marrow.....51–60, 122, 175
brain tumor initiating cell (BTIC).....37–48
cardiomyocyte.....111, 204, 242
embryonic fibroblasts.....133, 175
expansion 52, 91, 92
hair follicle.....92, 151, 203–225
hepatocyte-like cells.....132, 137
isolation 121–122
melanocytes 203–225
mesenchymal stem cells (MSCs) 92, 118,
120–122, 127, 162, 167, 168, 205
neural crest cells.....79–89
neural precursor cells (NPCs) 9–18
neural stem cells (NSCs) 9–18, 37,
39, 41, 47, 81, 83, 86, 163
outer root sheath.....203–225
peripheral neuron.....81, 83, 87–88
retinal pigment epithelial cells 183–192
side population (SP) cells.....51–60
stem cells
adipose derived stem or regenerative cells.....166
adult stem cells.....92, 117, 162, 204
dental pulp stem cells (DPSC) 91–114, 116–128
human embryonic stem cells
(hESCs) 80–85, 183, 184, 204, 225
human induced pluripotent stem cells
(hiPSCs) 131–139, 183–192
mouse embryonic stem cells (mESCs).....1–7, 144
rat induced pluripotent stem
cells (riPSCs) 143–149
satellite cells 63–76
Schwann cells 79–81, 83, 87, 89

spermatogonial 193–201

Culture

spheroid culture 134, 136–137
suspension culture.....51–60
three-dimensional (3D) culture 107, 131–139,
229–236

D

Development 1, 2, 9, 10, 12, 47, 63, 79, 118,
132, 149, 161–162, 166, 168, 169, 184, 193–201,
229–236

Differentiation

neurogenic molecules.....12
neurospheres 12, 17, 18
osteogenic 88, 111, 117–128

DPSC. *See* Dental pulp stem cells (DPSC)

E

Explants.....205, 210–221, 229–236

F

Flow cytometry.....39, 41–43, 48, 53, 57, 85, 108,
122, 168, 175–176, 184

H

Histology.....156, 168, 242, 245, 246

I

Immunohistochemistry (IHC) 14, 16–17,
39, 40, 45–47, 76, 88, 107, 109, 110, 118, 154,
156, 184, 193–201, 242, 245

Immunostaining 63–76, 149

L

Lentiviral vectors 145, 146, 149

Lesion

cortical trauma.....12
intramedullar 23–34
intrathecal.....23–34
spinal cord injury 11, 23–34
traumatic.....23–34

M

Morphogenesis 230, 233, 235

R

Regenerative medicine

 myocardium regeneration 244

 self-renewal 64, 183

T

Therapeutic targets 152

Tissue

 cardiac tissue engineering 239–246

 dental tissues 117–128

 embryonic epicardium 240

 pancreas 229–236

 skeletal muscle 51–60, 169

 skeletal muscle fiber 63–76

 skeletal myofiber 169, 245

 skin cutaneous wound healing 151–157

 skin regeneration 207

 spinal cord 9, 24, 25, 27, 28, 30,

 34, 163

Transcription factors 2, 143, 153, 194,

 206, 207

Transplantation 9–18, 23–34, 37, 38, 92, 119,

 164–169, 194

X

Xenograft 37–48