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Embryonic Stem Cell Protocols

Volume 2: Differentiation Models

SECOND EDITION

Edited by
Kursad Turksen

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Embryonic Stem Cell Protocols, Vol. 2

Second Edition

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Preface

The potentials, and hence popularity, of assessing embryonic stem (ES) cells in regenerative medicine applications is no longer a surprise to either scientists or the general public. This is clearly reflected in the ever-increasing publications in which ES cell biology and differentiation along diverse lineages appear in the academic as well as the popular press. It is also reflected in the intense interest in the isolation and characterization of ES cells from other species for preclinical studies. It therefore seemed timely to capture important advances in the field since the publication of the *Embryonic Stem Cells: Methods and Protocols* volume four years ago.

To provide an update and complement the original mouse ES cell book, I have focused the initial part of the first volume of the new series on ES cells recently isolated from other/nonmouse species. Second, the volumes contain numerous updates, more advanced approaches and completely new protocols for the use of ES cells in studies of diverse cell lineages. I believe that these two volumes will complement and expand the experimental repertoires of both experts and novices in the field. I would therefore like to take this opportunity to thank all of the contributors for their generosity and dedication in putting together their protocols. Without them, these volumes would not exist.

I am grateful to Dr. John Walker for his support and encouragement during the process. I would also like to thank several others at the Humana Press for their support: initially Elyse O'Grady and Craig Adams, and more recently Damien DeFrances. Also, I am grateful to Jennifer Hackworth for her wonderful support during the production of this volume.

I would also like to thank Jane Aubin and N. Urfe for their continuous support and encouragement as well as Tammy Troy who has once again been fantastic in helping to put together these volumes.

Kursad Turksen

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1

Neural Stem Sphere Method

Induction of Neural Stem Cells and Neurons by Astrocyte-Derived Factors in Embryonic Stem Cells In Vitro

Takashi Nakayama and Nobuo Inoue

Summary

We have developed a novel method to produce large numbers of neural stem cells and neurons directly from embryonic stem (ES) cells. The method is composed of three culture stages. In the first stage, floating ES cell colonies are cultured in astrocyte-conditioned medium to directly differentiate neural stem cells from ES cells, resulting in neural stem spheres. In the second stage, neural stem spheres are cultured as adhesive cells to produce neural stem cells by cell migration. In the third stage, neural stem cells are cultured as monolayers, which proliferate in the presence of fibroblast growth factor 2 and epidermal growth factor and differentiate into neurons in astrocyte-conditioned medium.

Key Words: Astrocyte-conditioned medium; embryonic stem cells; neural stem cells; neural stem sphere.

1. Introduction

Mouse embryonic stem (ES) cells derived from the inner cell mass in the blast stage embryo are pluripotent and are thus able to differentiate into any kind of cell. Many procedures exist to produce neural stem cells and neurons from ES cells, but each of these requires considerable time and many difficult steps ***(1,2)***. The protocol presented here, the neural stem sphere method, describes a method by which ES cells can be maintained and differentiated directly into neural stem cells and neurons. This method, which uses astrocyte-derived factors, provides large numbers of neural stem cells and neurons in a short time and without difficult steps ***(3)***. Starting with ES cells, neural stem cells can be expanded about 1000-fold, preserved by freezing, and differentiated into neurons in vitro ***(4)***. This procedure may be used to provide donor cells for neuronal transplantation in the therapy of neurodegenerative diseases. It may also be used

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to provide functional neurons, which may serve as excellent substitutes for tissue-derived primary cultured neurons in a wide range of experiments, including high-throughput screening of neuropharmacological agents. This protocol is truly valuable because it can be applied directly to primate ES cells with little modification.

2. Materials

2.1. Tissue Culture

1. Calcium- and magnesium-free phosphate-buffered saline (PBS): 8 g sodium chloride, 0.2 g potassium chloride, 1.15 g sodium monohydrogen phosphate (anhydrous), and 0.2 g potassium dihydrogen phosphate (anhydrous) in 1 L H_2O.
2. Gelatin type A from porcine skin (100 g; Sigma, St. Louis, MO; cat. no. G-1890) (*see* **Note 1**).
3. Dimethyl sulfoxide (DMSO) (100 mL; Sigma; cat. no. D-2650).
4. Dulbecco's modified Eagle's medium (DMEM; 500 mL; Gibco Invitrogen Corp., Grand Island, NY; cat. no. 11995-065 for ES cells and cat. no. 11965-092 for MEFs).
5. DMEM/nutrient mixture F-12 Ham (DMEM/F12) (500 mL; Sigma, cat. no. D-8437).
6. N2 supplement (5 mL; Gibco Invitrogen Corp., cat. no. 17502-048).
7. Neurobasal medium (500 mL; Gibco Invitrogen Corp., cat. no. 21103-049).
8. B-27 supplement (10-mL; Gibco Invitrogen Corp., cat. no. 17504-044).
9. Growth factor-reduced Matrigel basement membrane matrix (10 mL; BD Biosciences, San Jose, CA; cat. no. 356230) (*see* **Note 2**).
10. 100 mL 200 m*M* L-glutamine solution (100X; Sigma, cat. no. G-7513).
11. 500 mL fetal calf serum (FCS; Irvine Scientific, Santa Ana, CA; cat. no. 3000).
12. 500 mL horse serum (HS; Gibco Invitrogen Corp., cat. no. 16050-122).
13. 500 mL Knockout™ serum replacement (KSR; Gibco Invitrogen Corp., cat. no. 10828-028).
14. Leukemia inhibitory factor (LIF; ESGRO™ 10^6 U; Chemicon International Inc., Temecula, CA; cat. no. ESG1106).
15. 100 mL 10 m*M* minimum essential medium nonessential amino acids (NEAA) solution (100X; Gibco Invitrogen Corp., cat. no. 1140-050).
16. 100 mL β-mercaptoethanol (Sigma, cat. no. M-7552) (*see* **Note 3**).
17. 100 mL trypsin (10X; Sigma, cat. no. T-4549) (*see* **Note 4**).
18. Ethylenediaminetetraacetic acid (EDTA)-4Na (50 g; Wako, Tokyo, Japan; cat. no. 343-01883).
19. 100 mL penicillin-streptomycin (100X; Sigma, cat. no. P0781).
20. Mitomycin C (10 mg; Wako, cat. no. 134-07911) (*see* **Note 5**).
21. 35-mm polystyrene tissue culture dishes (Falcon, Franklin Lakes, NJ; cat. no. 353001).
22. 60-mm polystyrene tissue culture dishes (Falcon, cat. no. 353002).
23. 100-mm polystyrene tissue culture dishes (Falcon, cat. no. 353003).
24. Sumilon bacteriological 35-mm culture dishes (Sumitomo Bakelite Co. Ltd., Tokyo, Japan; cat. no. MS-1135R).
25. Polystyrene four-well multitissue culture dishes (Nunc, Roskilde, Denmark; cat. no. 176740).
26. 15-mL polypropylene conical tubes (Sumitomo Bakelite Co. Ltd., cat. no. MS-56150).
27. 50-mL polypropylene conical tubes (Sumitomo Bakelite Co. Ltd., cat. no. MS-56500).
28. Polyvinylidene fluoride 0.22-μm syringe filters (Millex® GV case of 50; Millipore, Bedford, MA; cat. no. SLGV 025).
29. 0.22-μm filter syringe filters for DMSO (Dimex® case of 50; Millipore, cat. no. SLLG 025LS).
30. Cryotube (2.0-mL tubes; Sarstedt, Nümbrecht, Germany; cat. no. 72.649.107S).

31. Microcoverglass (13 mm; Matsunami Glass Ind., Ltd., Osaka, Japan; cat. no. C013001).
32. Sterile syringes (1, 3, 5, 10, 20, and 50 mL; Terumo Corp., Tokyo, Japan).
33. Astrocyte-conditioned medium (50 mL; Nerve-Cell Culture System 50, Sumitomo Bakelite Co. Ltd., cat. no. MB-X9501).
34. 25 µg fibroblast growth factor 2 (FGF-2; R&D Systems Inc., Minneapolis, MN; cat. no. 234-FSE) (*see* **Note 6**).
35. 200 µg epidermal growth factor (EGF; R&D Systems, Inc., cat. no. 236-EG) (*see* **Note 6**).
36. Aspirator tube assembly (five sets; Sigma, cat. no. A-5177).
37. Capillary tubes (75 mm; Drummond Scientific Co., Broomall, PA; cat. no. 1-000-8000).

2.1.1. Media

1. Mouse embryonic fibroblasts (MEFs) are maintained in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin. To prepare 500 mL, combine 450 mL DMEM, 50 mL FCS, and 5 mL penicillin-streptomycin.
2. Mouse or rat astrocytes are maintained in DMEM supplemented with 10% HS and 1% penicillin-streptomycin. To prepare 500 mL, combine 450 mL DMEM, 50 mL HS, and 5 mL penicillin-streptomycin.
3. Conditioned medium: to prepare conditioned medium, astrocytes are cultured in serum-free medium consisting of DMEM/F12, 1% N2 supplement, and 1% penicillin-streptomycin (DMEM/F12/N2 supplement) (*see* **Subheading 3.1.5.2.)**.
4. HK ES cells are maintained in DMEM supplemented with 15% KSR, 1000 U/mL LIF, 1% NEAA, 100 µ*M* β-mercaptoethanol, and 1% penicillin-streptomycin (DMEM/KSR/LIF). To prepare 100 mL, combine 85 mL DMEM, 15 mL KSR, 100 µL LIF (1×10^5 U), 1 mL NEAA, 100 µL 100 m*M* β-mercaptoethanol, and 1 mL penicillin-streptomycin.
5. ES cell-derived neural stem cells are maintained in Neurobasal medium supplemented with 2% B-27, 20 ng/mL FGF-2, 20 ng/mL EGF, and 1% penicillin-streptomycin (Neurobasal B-27). To prepare 100 mL: combine 100 mL Neurobasal medium, 2 mL B-27, and 1 mL penicillin-streptomycin. FGF-2 and EGF are added directly to the cultures at final concentrations of 20 ng/mL each.
6. Freezing medium: 10% DMSO and 90% medium for MEF cells and 10% DMSO and 90% FCS for ES cells.

2.1.2. Tissue Culture

1. 37°C water bath.
2. Hemocytometer.
3. Disposable plastic pipets (5, 10, 25, and 50 mL).
4. Eppendorf pipets (2, 10, 100, and 1000 µL).
5. Tabletop centrifuge.
6. Liquid nitrogen storage tank.
7. Deep freezer (–80°C).
8. Inverted microscope equipped with phase contrast optics and camera.
9. Humidified 37°C, 5% CO_2 incubator.
10. Stereo dissection microscope.
11. Laminar flow cabinet.
12. Mouth-controlled glass capillary for picking up and transferring ES cell colonies and neural stem spheres: heating a glass pipet over a small gas or alcohol burner flame will cause it to melt. It can then be pulled to narrow the internal diameter as needed. An ampule cutter

can then be used to break the pipet, but it is not necessary to flame polish the opening at the tip. The capillaries can be sterilized by baking. Between a plastic mouthpiece and a glass capillary holder (aspirator tube assembly), a sterile 0.22-µm filter is inserted to trap saliva.

2.2. Immunofluorescence

1. Paraformaldehyde (500 g; Sigma, cat. no. P6148) (*see* **Note 7**).
2. Perma Fluor (30 mL; Shandon Lipshaw Inc., Pittsburgh, PA; cat. no. 434990).

3. Methods

3.1. Tissue Culture

3.1.1. Freezing Cells

1. Wash cells several times in PBS (–) and treat with 0.25% trypsin-EDTA solution for 5 min. Trypsinization is stopped by the addition of FCS.
2. Cells are collected into a conical tube and counted with a hemocytometer.
3. Cells are centrifuged at 700*g* for 5 min, and the pellet is resuspended into an appropriate amount of freezing medium.
4. Cells are aliquoted (0.5 or 1 mL) into cryotubes.
5. The cryotubes are transferred to a styrene foam box, which is placed overnight in a –80°C freezer.
6. The tubes are transferred to a liquid nitrogen container for long-term storage.

3.1.2. Thawing Cells

1. Remove the cryotube.
2. Float in a water bath at 37°C and allow the contents of the tube to melt.
3. Swab the tube with 70% ethanol to sterilize the outside.
4. To dilute the DMSO, transfer the cell suspension into several milliliters of culture medium in a 15-mL centrifuge tube.
5. Collect the cells by centrifugation at 700*g* for 5 min.
6. Suspend in fresh culture medium.
7. Transfer to a tissue culture dish.

3.1.3. Fibroblast Feeder Layer

ES cells require a feeder layer to maintain a pluripotent state. The feeder layers are established cell line (STO fibroblasts) or primary MEFs (*see* **Note 8**).

3.1.3.1. Preparation of MEFs

1. Sacrifice pregnant mice at 13–14 d; remove the embryos and place them in 100-mm dishes containing PBS (–).
2. Using forceps and scissors, remove the head and internal organs.
3. Wash several times in PBS (–).
4. Mince the carcasses finely with dissecting scissors.
5. Transfer to 50-mL centrifugation tubes.
6. Pellet the tissue pieces by centrifugation at 700*g* for 5 min.
7. Aspirate the supernatants, add 1 mL 0.25% trypsin-EDTA solution per embryo and incubate at 37°C for 20 min with shaking.
8. Stop the trypsinization by adding 1/10 the volume of FCS and dissociate the tissue by passing through a 10-mL disposable pipet.

9. Centrifuge the cell suspension at 700*g* for 5 min and remove the supernatant.
10. Resuspend the cells in 20 mL fresh culture medium per embryo.
11. Plate 10 mL cell suspension into each 100-mm dish.
12. Culture for several days until the cells reach confluency.
13. Cryopreserve MEFs as the primary passage: freeze the MEF in a 100-mm dish by suspending the cells in 1 mL freezing medium in a vial (*see* **Subheading 3.1.1.**).
14. To prepare an MEF feeder layer, expand MEFs only to the third passage.

3.1.3.2. Maintenance of MEFs

1. Thaw one vial of MEF primary clone and add 100 mL fresh culture medium.
2. Plate 10 mL onto each 100-mm dish.
3. Within 1 wk, dishes should be confluent (secondary clone).

3.1.4. ES Cells in Culture

3.1.4.1. Mitomycin C Treatment of MEFs for ES Cells

Nonproliferating MEFs are necessary to culture ES cell clones, including our ES cell line HK. To stop the proliferation of MEF, the cells should be treated with mitomycin C.

1. Remove 5 mL culture medium from an almost-confluent 100-mm dish of proliferating MEFs (*see* **Subheading 3.1.3.2.**).
2. Add 50 µL 1 mg/mL mitomycin C solution, then agitate.
3. Incubate in a 37°C, 5% CO_2 incubator for 2–3 h.
4. Wash mitomycin C-treated MEFs several times in PBS (–) and trypsinize.
5. Harvest the dissociated cells by centrifugation at 700*g* for 5 min.
6. Resuspend the mitomycin C-treated MEFs in freezing medium for cryopreservation.
7. Freeze the mitomycin C-treated MEFs from one 100-mm dish in one vial. This should be sufficient for preparing two 60-mm plates.

3.1.4.2. Thawing Mitomycin C-Treated MEFs

1. Precoat the culture dishes with gelatin. Spread 1 mL 0.1% gelatin solution onto the surface of a 60-mm culture dish for 1 h at room temperature.
2. Aspirate the gelatin solution and rinse once with 1 mL culture medium.
3. Thaw mitomycin C-treated MEFs and transfer the entire contents of a cryotube into 7 mL culture medium in a sterile tube and mix gently. Plate 4 mL cell suspension onto each gelatin-coated 60-mm dish. These cells can be used the next day as a feeder layer for ES cells.

3.1.4.3. Maintenance of ES Cells

To subculture ES cells, it is necessary to expand the number of ES cells.

1. Thaw one cryotube of ES cells in a water bath at 37°C and add the cells (10^5 cells in 0.5 mL freezing medium) to 10 mL DMEM in a 15-mL conical tube.
2. Collect the cells by centrifugation at 700*g* for 5 min and remove the supernatant.
3. Resuspend the cells in 8 mL DMEM/KSR/LIF.
4. Aspirate the culture medium from the MEF feeder layer. Plate 4 mL ES cell suspension onto the feeder layer in each 60-mm dish.
5. Change the medium every other day and culture until ES cells become confluent.
6. Make frozen ES cell stocks (*see* **Subheading 3.1.1.**) (*see* **Note 9**).

3.1.5. Astrocytes in Culture

To produce astrocyte-conditioned medium, rat astrocyte cultures are prepared using the following procedure (*5*).

3.1.5.1. Isolation of Astrocytes

1. Sacrifice pregnant rats at 19–20 d and remove the pups.
2. Decapitate a pup, remove the brain from the skull, and transfer into PBS (–) in a culture dish.
3. Remove the meninges, cerebellum, and brain stem.
4. Cut the cerebra into small pieces.
5. Transfer the tissue to a 15-mL conical tube, add 10 mL 0.25% trypsin-EDTA, and incubate at 37°C for 20–30 min with shaking.
6. Stop the trypsinization by adding 1 mL FCS and collect the cells by centrifuging at 700*g* for 5 min.
7. Suspend the cells in DMEM supplemented with serum.
8. Plate at 1–5 × 10^5 cells/mL.
9. Change the culture medium every 2 d.
10. When the culture reaches confluency, collect the astrocytes and freeze until use (*see* **Note 10**).

3.1.5.2. Production of Astrocyte-Conditioned Medium (*see* **Note 11**)

1. Thaw a vial of frozen astrocytes.
2. Add to 10 mL of DMEM/serum in a 15-mL conical tube and collect the cells by centrifugation at 700*g* for 5 min.
3. Plate at 1–5 × 10^5 cells/mL.
4. When the culture becomes confluent, wash astrocytes twice with plain DMEM to remove serum.
5. Add an appreciable amount of DMEM/F12/N2 supplement.
6. Leave for at least 3 d and remove the supernatant, defined as astrocyte-conditioned medium (*see* **Note 12**).

3.1.6. Differentiation of ES Cells Into Neural Stem Cells and Neurons in Neural Stem Spheres

Differentiation of ES cells into neural stem cells and neurons consists of three stages. The first stage consists of culturing pluripotent ES cells. The second consists of culturing ES cells, neural stem cells, and cells at a transitional phase of differentiation. The third consists of culturing neural stem cells and neurons.

3.1.6.1. Formation of ES Cell Colonies

To make large ES cell colonies (300–500 μm in diameter), the number of ES cells plated onto a 60-mm feeder layer must be less than 250–300 (*see* **Note 13**).

1. Thaw one vial of ES cells (5 × 10^2 cells in 0.5 mL freezing medium).
2. Transfer to 10 mL DMEM in a 15-mL conical tube and centrifuge at 700*g* for 5 min.
3. Aspirate the supernatant, resuspend in 8 mL DMEM/KSR/LIF and inoculate 4 mL cell suspension onto a 60-mm feeder layer.
4. The following day, change the culture medium.
5. Change the culture medium every other day. When ES cell colonies have become large, change the culture medium every day.
6. After 7–9 d in culture, ES cell colonies reach 300–500 μm in diameter and may be picked.

3.1.6.2. Formation of Neural Stem Spheres—Floating Culture

During culture for 4 d, floating ES cell colonies become neural stem spheres and develop a core. A dense core is visible under a stereo dissection microscope.

1. Wash ES cells twice with DMEM prewarmed to 37°C.
2. Select undifferentiated smooth ES cell colonies using a stereo dissection microscope.
3. Place the tip of a mouth-controlled glass capillary at the edge of an ES cell colony and pick it up.
4. Using the mouth-controlled glass capillary, transfer the colony to a 35-mm nonadherent bacteriological dish containing 2 mL DMEM.
5. Using the same method, collect all the floating ES cell colonies in a dish and wash in DMEM to remove any cell debris.
6. Using the mouth-controlled glass capillary, transfer the colonies to a 35-mm bacteriological dish containing 2 mL astrocyte-conditioned medium and maintain fewer than 20 colonies per dish. Floating cultures require at least 4 d to develop spheres with dense cores (*see* **Note 14**).

3.1.6.3. Adhesion of Neural Stem Spheres and Neural Formation

1. Apply 1 mL diluted Matrigel onto a 60-mm dish with a 1-mL sterile tip using an Eppendorf pipet, spread out without bubbling, and transfer the solution to the next dish.
2. Leave for 1 h in a 37°C, 5% CO_2 incubator.
3. Aspirate the Matrigel solution and rinse each dish in 2 mL DMEM.
4. Aspirate the DMEM and add 4 mL astrocyte-conditioned medium prewarmed to 37°C.
5. Using a stereo dissection microscope, select cored neural stem spheres proliferating during floating culture and transfer to a Matrigel-coated dish. Transfer about 10 neural stem spheres to each 60-mm dish for culture and differentiation. Neural stem spheres adhere to the substrate within several hours (*see* **Notes 15** and **16**).

3.1.7. Expansion of Neural Stem Cells

Expand neural stem cells during neural stem sphere formation by culture in astrocyte-conditioned medium supplemented throughout with FGF-2 and EGF (*see* **Note 17**).

1. Pick ES cell colonies as described in **Subheading 3.1.6.2.**
2. Wash in DMEM.
3. Transfer to 2 mL astrocyte-conditioned medium in a 35-mm bacteriological dish using a mouth-controlled glass capillary. Culture fewer than 20 colonies per dish.
4. Add stock solution of FGF-2 and EGF to astrocyte-conditioned medium to final concentrations of 20 ng/mL each.
5. Add FGF-2 and EGF every other day.
6. Floating cultures require at least 4 d to develop neural stem spheres with expanded neural stem cells (*see* **Note 18**).

3.1.7.1. Migration of Neural Stem Cells From Adherent Neural Stem Spheres

1. Coat a 60-mm dish with 50-fold diluted Matrigel solution as described in **Subheading 3.1.6.3.**
2. Wash once in DMEM.
3. Add 4 mL Neurobasal B-27 prewarmed to 37°C.
4. Transfer neural stem cells expanded from neural stem spheres to the adherent culture dish.
5. Maintain fewer than 10 spheres per 60-mm dish.

6. Add FGF-2 and EGF at 20 ng/mL each and thereafter every other day (*see* **Note 19**).
7. Change medium every 2–4 d, depending on the degree of neural stem cell expansion (*see* **Notes 20** and **21**).

3.1.7.2. Cryopreservation of ES Cell-Derived Neural Stem Cells

1. After 7 d, over 10^5 neural stem cells migrate from a single adherent neural stem sphere (*see* **Note 22**).
2. Remove neural stem spheres (*see* **Note 23**).
3. Transfer detached neural stem spheres to a new dish precoated with Matrigel and containing Neurobasal B-27 medium and add FGF-2 and EGF (*see* **Note 24**).
4. Wash twice in PBS (–).
5. Add 2 mL 0.05% trypsin-EDTA (fivefold dilution from 0.25% trypsin-EDTA; *see* **Note 4**) per 60-mm dish, incubate for 5 min at 37°C and add 0.5–1 mL FCS to stop trypsinization.
6. Collect cells and count them.
7. Centrifuge cells at 700*g* for 5 min.
8. Aspirate the supernatant and resuspend the cells in 10% DMSO-90% FCS at 5–10 × 10^5 cells/mL.
9. Dispense 1-mL aliquots into cryotubes.
10. Freeze as described in **Subheading 3.1.1.** (*see* **Note 25**).

3.1.7.3. Thawing ES Cell-Derived Neural Stem Cells

1. Thaw a frozen cryotube of ES cell-derived neural stem cells.
2. Add to 10 mL DMEM in a 15-mL conical tube and pellet by centrifugation at 700*g* for 5 min.
3. Resuspend in Neurobasal B-27 medium at a density of 1–5 × 10^5 cells/mL.
4. Inoculate 4 mL onto a 60-mm Matrigel-coated dish.
5. Add FGF-2 and EGF at 20 ng/mL each.
6. Every other day, add FGF-2 and EGF.
7. Change the medium every 2–4 d, depending on the degree of neural stem cell proliferation (*see* **Fig. 1F–H**).
8. Before the culture becomes confluent, subculture or freeze neural stem cells by dissociation in 0.05% trypsin-EDTA (*see* **Subheading 3.1.7.2.**).

3.1.7.4. Differentiation of ES Cell-Derived Neural Stem Cells

Frozen ES cell-derived neural stem cells can be differentiated into neurons by culture in astrocyte-conditioned medium (*see* **Note 26**).

1. Habituate freshly thawed ES cell-derived neural stem cells in Neurobasal B-27 medium containing FGF-2 and EGF for several days.
2. When the culture reaches the desired density, induce differentiation by changing the medium to astrocyte-conditioned medium (*see* **Notes 27** and **28**).

3.2. Analysis of Differentiation

Immunofluorescence staining and RT-PCR *(3,4)* can be used to determine stages of cell differentiation.

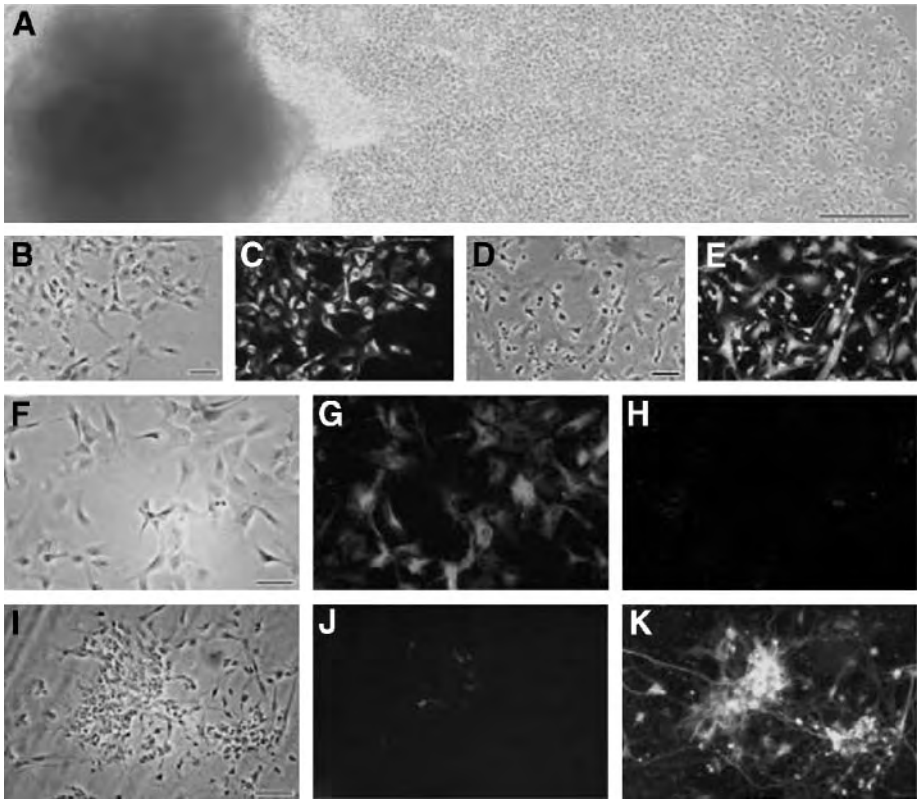

Fig. 1. Adhesion cultures of neural stem spheres and neural stem cells. (**A–E**) Neural stem spheres after culture in astrocyte-conditioned medium supplemented throughout with fibroblast growth factor (FGF)-2 and epidermal growth factor (EGF) attached onto Matrigel-coated dishes and subsequently cultured in Neurobasal B-27 medium supplemented with FGF-2 and EGF for 7 d. (**A**) Phase contrast micrograph at low magnification. Many neural stem cells migrate from and around the attached sphere. (**B**) High-magnification micrograph of migrated neural stem cells. (**C**) Immunostaining of nestin showing that all the migrated cells are neural stem cells. (**D**,**E**) Differentiation of migrated neural stem cells by changing the medium from Neurobasal B-27 to astrocyte-conditioned medium and withdrawing growth factors. (**D**) Phase contrast micrograph. Many neural stem cells differentiate into neurons with neurites after 5 d in culture. (**E**) Immunostaining of neurofilament H, a marker of neurons; many cells are positively stained. (**F–H**) Proliferation of cryopreserved neural stem cells. (**F**) Phase contrast micrograph. (**G**) Nestin staining profile. (**H**) NF-H staining profile. (**I–K**) Differentiation of cryopreserved neural stem cells. Proliferated neural stem cells are induced to differentiate into neurons by changing the medium from Neurobasal B-27 to astrocyte-conditoned medium and withdrawing growth factors. (**I**) Phase contrast micrograph. (**J**) Nestin staining profile. (**K**) NF-H staining profile. Bars represent 100 μm in (**A**) and 50 μm in (**B**), (**D**), (**I**), and (**J**).

3.2.1. Immunofluorescence Staining

3.2.1.1. Culture on Glass Cover Slips

To determine cellular morphology and to localize antigens, cells at each developmental stage are cultured on Matrigel-coated 13-mm cover slips.

1. Place a 13-mm clean cover slip (sterilized by baking) in each well of a four-well multidish.
2. Coat cover slips with 50-fold diluted Matrigel solution as described in **Subheading 3.1.6.3.**
3. Aspirate and rinse once in 0.5 mL DMEM.
4. Inoculate 0.5 mL cells at a density of 1–5 × 10^5 cells/mL.

3.2.1.2. Staining

Fluorescence labeling is useful for identifying specific cell types (*see* **Note 29**).

1. Fix cells grown on 13-mm cover slips with 0.5 mL 4% paraformaldehyde in PBS (–) for 15 min.
2. Rinse twice in PBS (–).
3. Permeabilize by incubation with 0.5% Triton X-100 in PBS (–) for 5 min.
4. Rinse twice in PBS (–).
5. Block the specimens with 10% bovine serum albumin (BSA) or 10% normal goat serum in PBS (–) for 1–2 h at room temperature.
6. Add the first antibody at an adequate dilution in PBS (–) containing 1% BSA or 1% normal goat serum.
7. Incubate for 2 h at room temperature or overnight at 4°C.
8. Rinse three times in PBS (–) over 10 min.
9. Add the fluorescence labeled secondary antibody at an adequate dilution in PBS (–) containing 1% BSA or 1% normal goat serum.
10. Incubate for 2 h at room temperature.
11. Rinse three times in PBS (–) over 10 min.
12. Rinse once in water for 1 min (*see* **Note 30**).
13. Add a small drop of mounting medium (Perma Fluor) on a slide glass.
14. Invert the cover slips and mount onto glass slides (sample side down), avoiding air bubbles.
15. After drying, remove excess mount medium with filter paper.
16. Seal each cover slip by spreading a small volume of nail polish at its edge to avoid air-drying.
17. Examine under a fluorescent microscope equipped with a photographic apparatus or under a confocal laser-scanning microscope.

4. Notes

1. To prepare 0.1% gelatin solution, add 100 mg gelatin in 100 mL H_2O, dissolve, and sterilize by autoclave.
2. To prepare 1 mL diluted Matrigel, sufficient to coat four or five 60-mm dishes, combine 20 µL stock Matrigel solution with 980 µL DMEM chilled on ice.
3. The stock solution of 100 m*M* β-mercaptoethanol is made by diluting 70 µL β-mercaptoethanol with 10 mL H_2O; it is then filter-sterilized, divided into aliquots, and stored at –20°C.
4. To prepare 0.25% trypsin-EDTA solution, mix 1 vol 10X trypsin, 0.5 vol 10.6 m*M* EDTA-4Na (dissolved in water and sterilized with a 0.22-µm filter), and 8.5 vol PBS (–).

5. The stock solution of 1 mg/mL mytomycin C is made by dissolving 5 mg mytomycin C in 5 mL H_2O; it is then filter-sterilized. The 1-mL aliquots are stored at –20°C in dark tubes to protect from light.
6. FGF-2 and EGF are dissolved in sterile PBS (–) containing 0.1% BSA at a concentration of 20 µg/mL and stored at –80°C until use.
7. To prepare 4% paraformaldehyde in PBS, dissolve 8 g paraformaldehyde in 100 mL H_2O at 60°C and add a few drops 1 *N* NaOH. After cooling, mix with 2X PBS (–).
8. Primary MEFs are easy to prepare and are not expensive.
9. For subculturing of ES cells, store 10^5 cells in 0.5 mL freezing medium in each cryotube. For ES cell differentiation experiments, store 5×10^2 cells in 0.5 mL freezing medium in each cryotube.
10. This culture contains mainly type 1 astrocytes as well as other glial cell types. The latter cells are less adherent and can be removed by shaking the culture flask ***(6)***, but it is not necessary in this experiment.
11. To make it easier to try this method, we recommend purchasing commercially available astrocyte-conditioned medium because it is difficult to prepare properly. Good-quality astrocyte-conditioned medium can be purchased from Sumitomo Bakelite Company, Tokyo, Japan (*see* **Subheading 2.1.**).
12. Filtrate recovered astrocyte-conditioned medium and store at –80°C. To promote neural stem sphere formation, use astrocyte-conditioned medium in a 50:50 mixture with fresh DMEM/F12/N2 supplement.
13. The size of colony is important to get good results.
14. Each cored sphere is a concentric stratiform structure, with the periphery of the neural stem cells expressing nestin. These neural stem spheres are composed of a periphery of neural stem cells, a core of ES cells, and an intermediate layer of cells making a transition from ES to neural stem cells (*see* **Fig. 2**).
15. Usually, neuronal neurites extend from adherent neural stem spheres on the next day.
16. Addition of growth factors or cytokines to astrocyte-conditioned medium is useful for determining the effects of specialization on neural phenotype ***(7)***.
17. Addition of FGF-2 alone is less effective.
18. Neural stem cells expanded from neural stem spheres are composed of a core of ES cells, with the rest nestin-positive neural stem cells (*see* **Fig. 3**).
19. Usually, neural stem cells begin to migrate from adherent neural stem cells on the following day.
20. After 7 d, nestin-positive neural stem cells migrating to the head reach 500–600 µm from the adherent neural stem spheres (*see* **Fig. 1A–C**).
21. These migrated neural stem cells can be induced to differentiate into neurons by changing the medium from Neurobasal B-27 to astrocyte-conditioned medium and withdrawing FGF-2 and EGF (*see* **Fig. 1D**,**E**).
22. These neural stem cells can be stored by freezing and differentiated after thawing.
23. Adherent neural stem spheres may contain undifferentiated ES cells, so they are detached from the center of migrated neural stem cells using a mouth-controlled glass capillary.
24. These spheres become new seeds of migrating neural stem cells. The transplanting of neural stem spheres can be repeated several times.
25. Frozen stocks of neural stem cells can be safely stored in liquid nitrogen and shipped anywhere in dry ice.
26. Be sure not to culture freshly thawed ES cell-derived neural stem cells directly in astrocyte-conditioned medium. Rather, conditioning in a previously used proliferation medium is

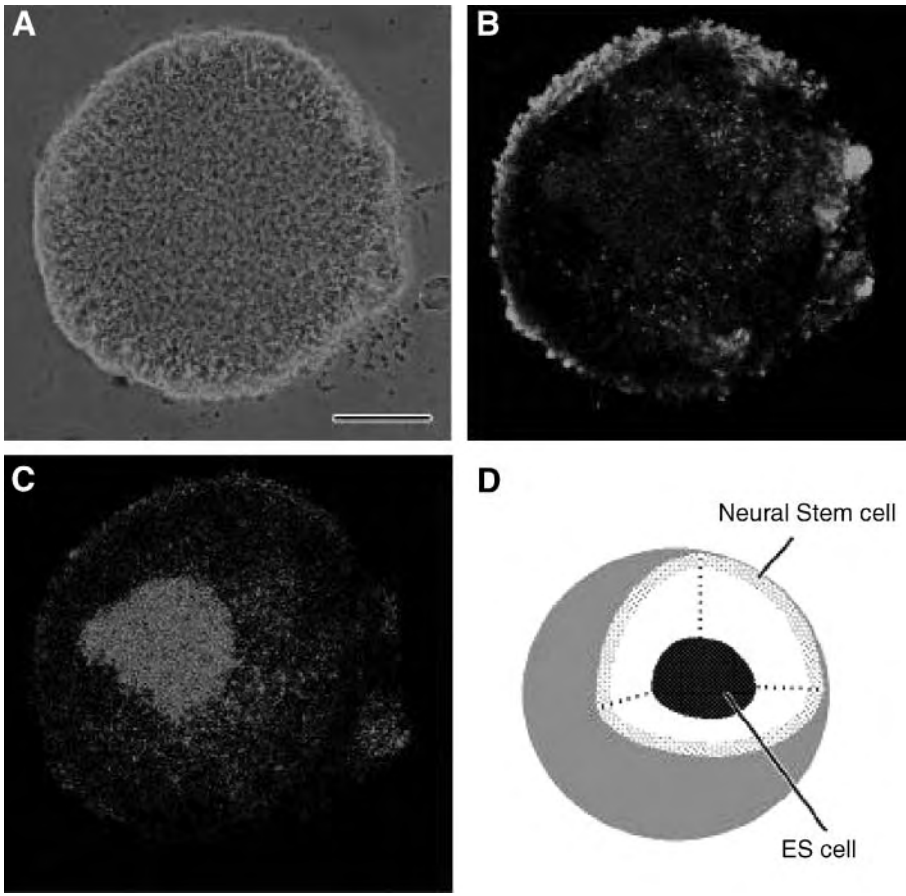

Fig. 2. Structure of a neural stem sphere after floating culture in astrocyte-conditioned medium for 4 d. (**A**) Phase contrast micrograph of a neural stem sphere. (**B**) Immunostaining of nestin-positive neural stem cells by confocal microscopy. Neural stem cells are present at the periphery of the neural stem sphere. (**C**) Immunostaining of BrdU-positive embryonic stem (ES) cells by confocal microscopy. Actively proliferating $BrdU^+$ ES cells are present at the core of the neural stem sphere. (**D**) Model of a neural stem sphere from focal images. The concentric stratiform structure is composed of at least three layers: a periphery of neural stem cells, a core of self-renewing ES cells, and an intermediate layer of cells at a transitional stage of differentiation from ES to neural stem cells. Bar represents 100 µm.

necessary. Proliferative neural stem cells in subculture, however, may be differentiated directly by changing the culture medium.

27. Usually, ES cell-derived neural stem cells start differentiating to neurons in 3–5 d (*see* **Fig. 1I–K**).
28. Additional time is required until almost all the cells develop neuronal morphology and express neuronal cell markers, such as neurofilament protein.

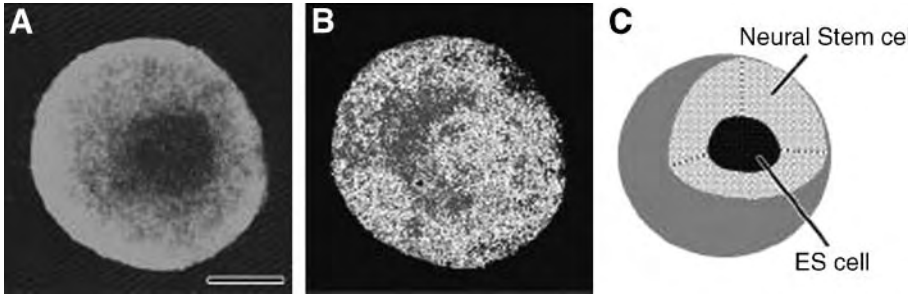

Fig. 3. Structure of a neural stem sphere cultured in astrocyte-conditioned medium supplemented throughout with fibroblast growth factor (FGF)-2 and epidermal growth factor (EGF). (**A**) Immunostaining of nestin-positive neural stem cells by confocal microscopy. Addition of FGF-2 and EGF increases the number of neural stem cells, which are present throughout the neural stem sphere, except for the core. (**B**) Immunostaining of BrdU-positive embryonic stem (ES) cells by confocal microscopy. Almost all cells are proliferative and BrdU positive. (**C**) Model of a neural stem sphere in expanded neural stem cell culture. Its concentric structure is composed of two parts: a periphery of neural stem cells and a core of ES cells. Bar represents 100 µm.

29. The growing cells are fixed in paraformaldehyde solution. Alexa Fluor (Molecular Probes Inc.) is less susceptible to fading, making it useful for fluorochromes.
30. This step can be omitted.

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2

Generation and Characterization of Oligodendrocytes From Lineage-Selectable Embryonic Stem Cells In Vitro

Nathalie Billon, Christine Jolicoeur, and Martin Raff

Summary

Oligodendrocytes develop from proliferating oligodendrocyte precursor cells (OPCs), which arise in germinal zones, migrate throughout the developing white matter and divide a limited number of times before they terminally differentiate. Thus far, it has been possible to purify OPCs only from the rat optic nerve, but the purified cells cannot be obtained in large enough numbers for conventional biochemical analyses. Moreover, the central nervous system stem cells that give rise to OPCs have not been purified, limiting the ability to study the earliest stages of commitment to the oligodendrocyte lineage. Pluripotent mouse embryonic stem (ES) cells can be propagated indefinitely in culture and induced to differentiate into various cell types. We describe protocols for culture conditions in which neural precursor cells, OPCs, and oligodendrocytes can be efficiently produced from genetically modified ES cells. This strategy should be useful for study of the intracellular and extracellular factors that direct central nervous system stem cells down the oligodendrocyte pathway and influence subsequent oligodendrocyte differentiation. It may also be useful for producing OPCs and oligodendrocytes from human ES cells for cell therapy and drug screening.

Key Words: Differentiation; ES cells; genetic selection; oligodendrocyte.

1. Introduction

Oligodendrocytes are postmitotic cells that myelinate axons in the vertebrate central nervous system (CNS). Like the majority of other cells in the CNS, oligodendrocytes are generated from pluripotent neural precursor cells of the neural tube, which itself derives from the embryonic ectoderm. Oligodendrocyte development begins in the embryo when proliferating oligodendrocyte precursor cells (OPCs) arise in restricted regions of the ventral ventricular zone of the developing brain and spinal cord *(1–5)*. The restriction of OPC development to these regions depends on both localized positive and negative signals *(6,7)*. One such positive signal is Sonic hedgehog (Shh), which is secreted by both the floor plate and the notochord along

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the rostrocaudal axis *(8–10)*. In the mouse, OPC markers, such as the *Olig1* and *Olig2* transcription factors *(11,12)*, the platelet-derived growth factor receptor (PDGFR)-α *(3,13)*, and the NG2 proteoglycan *(14)* are all expressed by embryonic age 13 (E13) *(6)*.

After they have arisen in germinal zones of the brain and the spinal cord, OPCs migrate throughout the developing white matter, where they divide a limited number of times, largely in response to PDGF *(15)*. They then stop moving, withdraw from the cell cycle, and terminally differentiate into myelinating oligodendrocytes, which express galactocerebroside (GC) *(16,17)*. The first GC-expressing oligodendrocytes seem to appear in the mouse CNS around E17 *(18)*.

The differentiation of OPCs into oligodendrocytes is better understood than the initial commitment of neural precursors to the oligodendrocyte lineage. This is mostly because of the development of a powerful in vitro system in which purified OPCs isolated from the perinatal rat optic nerve can proliferate and differentiate in serum-free cultures on the same schedule as they do in vivo *(19)*. Using this system, it has been shown that the OPCs have an intrinsic timer that regulates when they stop dividing and differentiate, and that at least two kinds of extracellular signals seem to be required for the timer to operate normally: PDGF *(20–22)* and thyroid hormone *(23–26)*. This cell model has been an invaluable tool in the identification of a number of intracellular mechanisms that regulate OPC differentiation *(27–32)*.

Although studies of purified rat OPCs have provided important insights into the control of OPC differentiation, the cells cannot be purified in large enough numbers for conventional biochemical analyses. Moreover, the neural precursor cells that give rise to OPCs have not been purified, making it difficult to study the earliest stages of OPC specification.

Mouse embryonic stem (ES) cells are proliferating, pluripotent stem cells that have been isolated from the epiblast of blastocyst stage mouse embryos *(33–35)*. They can be propagated indefinitely in culture in the presence of leukemia inhibitory factor (LIF) *(36,37)*. When transplanted into a mouse blastocyst, ES cells integrate into the embryo and contribute to all cell lineages, including germ cells *(38)*. If ES cells are cultured without LIF on a nonadherent surface, then they aggregate to form embryoid bodies (EBs), in which the cells form ectodermal, mesodermal, and endodermal derivatives *(39)*. ES cells can be produced in large numbers, easily genetically modified, and induced to differentiate into various CNS cell types in vitro *(40–43)*. They should therefore provide a powerful system for studying the early events of neural development.

Here, we describe protocols for culture conditions in which neural precursor cells, OPCs, and oligodendrocytes can be efficiently produced from genetically modified ES cells. This strategy should be useful for study of the intracellular and extracellular factors that direct CNS stem cells down the oligodendrocyte pathway and influence subsequent oligodendrocyte differentiation. It may also be useful for producing OPCs and oligodendrocytes from human ES cells for cell therapy and drug screening. Oligodendrocytes derived from mouse ES cells have already been shown to remyelinate axons in rodent models of demyelinating diseases *(43,44)*.

2. Materials

2.1. Tissue Culture

The tissue culture facility for ES cell culturing requires the following:

1. Humidified incubator at 37°C and 5% CO_2.
2. Laminar flow cabinet.
3. 37°C water bath.
4. Centrifuge.
5. Inverted microscope.
6. Coulter cell counter Z2 series.
7. Plastic pipets designated for tissue culture (5, 10, and 25 mL).
8. BD Falcon™ conical centrifuge tubes (15 and 50 mL; BD Biosciences, Cambridge, UK; cat. no. 352086 and 352196).

2.1.1. Maintenance of ES Cells

1. Genetically engineered ES cells ***(45)***.
2. 2% gelatin (bovine skin, Sigma-Aldrich, Gillingham, UK; cat. no. G1890). Add 2 g gelatin in 100 mL H_2O. Autoclave and store at 4°C.
3. Polystyrene, 25-cm^2, canted, phenolic Costar flasks (Fisher Scientific, Leicestershire, UK; cat. no. 430372).
4. Phosphate-buffered saline-ethylenediaminetetraacetic acid (PBS-EDTA): combine 8 g NaCl, 0.2 g KCl, 1.15 g disodium hydrogen orthophosphate, and 0.20 g potassium dihydrogen orthophosphate in distilled water. Adjust to pH 7.2 and complete to 1 L. Add 0.374 g EDTA. Autoclave.
5. 1X Trypsin (0.025%): add 5 mL trypsin 2.5% (Invitrogen Ltd., Paisley, UK; cat. no. 15090-046) to 500 mL PBS-EDTA. Store at 4°C.
6. Dulbecco's modified Eagle's medium (DMEM) (500-mL bottle; Invitrogen, cat. no. 31966 021).
7. Bovine serum (Invitrogen, cat. no. 26170043).
8. Penicillin-streptomicin-glutamine (PSG) (100X; Invitrogen, cat. no. 10 378-016).
9. Glasgow's modified Eagle's medium (GMEM, BHK21) (500-mL bottle; Invitrogen, cat. no. 21710-025).
10. Fetal calf serum (FCS) screened for ES cell growth (PAA Laboratories Gmbh, Somerset, UK; cat. no. A15-649) (*see* **Note 1**).
11. β-Mercaptoethanol (Sigma, cat. no. G9391) stock solution: add 100 β-mercaptoethanol to 14.1 mL H_2O. Prepare fresh.
12. 10 m*M* nonessential amino acids (100X; Invitrogen, cat. no. 11140-035).
13. 100 m*M* sodium pyruvate (100X; Invitrogen, cat. no. 11360-039).
14. 7% sodium bicarbonate (Invitrogen, cat. no. 25080-060).
15. 10^7 U/mL LIF (Chemicon, Hampshire, UK; cat. no. ESG1106).
16. Neutralization medium: for 500 mL, combine 500 mL DMEM, 50 mL bovine serum, and 5 mL PSG 100X.
17. ES cell medium: for 500 mL, combine 420 mL GMEM, 50 mL FCS, 5 mL PSG 100X, 0.5 mL β-mercaptoethanol stock solution, 5 mL 100X nonessential amino acid, 5 mL 100X sodium pyruvate, and 13.2 mL 7.5% sodium bicarbonate. For exponential culture, add LIF to a final concentration of 10^3 U/mL ($1/10^4$ dilution).

2.1.2. Differentiation of ES Cells Into the Oligodendrocyte Lineage

2.1.2.1. Coating of the Plates

1. Falcon polystyrene flasks (25 cm^2; BD Bioscience, cat. no. 353009).
2. Nunc four-well plates (Fisher, cat. no. 176740).
3. 1 mg/mL poly-D-lysine (PDL): dissolve 5 mg PDL (Sigma, cat. no. P 6407) in 5 mL H_2O. Aliquot (0.2 mL) and store at –20°C.
4. 1 mg/mL laminin (Sigma, cat. no. L 2020). Aliquot (0.2 mL) and store at –20°C.

2.1.2.2. Formation and Selection of Neural Precursors

1. 10-cm Petri dishes (BD Biosciences, cat. no. 351029).
2. Universal containers for EBs (Bibby Sterilin, Staffordshire, UK; cat. no. 128PYR).
3. All-*trans* retinoic acid (RA), 5000X (5×10^{-3} *M*): dissolve RA (Sigma, cat. no. R2625) in dimethyl sulfoxide. Aliquot (30 µL) and keep at –20°C away from light. Use once and discard.
4. Neural basal medium (Invitrogen, cat. no. 20103-049).
5. 4X trypsin (0.1%): add 20 mL 2.5% trypsin (Invitrogen, cat. no. 15090-046) to 500 mL PBS-EDTA. Store at 4°C.
6. Dulbecco's modified Eagle medium/F12 (DMEM/F12) (500-mL bottle; Invitrogen, cat. no. 31331 028).
7. 50X B27 (Invitrogen, cat. no. 17504).
8. 100X N2 (Invitrogen, cat. no. 17502).
9. 2000X geneticin sulfate (G-418) (200 mg/mL): dissolve G418 (Roche Diagnostics, Hertfordshire, UK; cat. no. 1464990) in sterile water to have 200 mg/mL active units (the activity is written on each tube of G418). Aliquot (0.5 mL) and store at –20°C.
10. 1000X fibroblast growth factor (FGF) (20 µg/mL): dilute 10 µg human FGF-2 (Peprotech, London, UK; cat. no. 100-18B) in 0.5 mL 0.01 *M* HCl. Aliquot (25 µL) and keep at –80°C.
11. 1000X ganciclovir (2.5 m*M*): dissolve 6.4 mg ganciclovir (Sigma, cat. no. G2536) in 10 mL DMEM. Sterilize through a 0.2-µm filter, aliquot (1 mL), and keep at –20°C.
12. DMEM/F12+N2 medium: add 1 mL N2 to 100 mL DMEM-F12.
13. Neural basal+B27 medium: add 2 mL B27 to 100 mL neural basal medium.
14. Neural differentiation medium: combine DMEM/F12+N2 medium and neural basal+B27 medium in equal volumes (1:1) to obtain F12/N2/NB/B27 medium. This medium can be kept at 4°C for up to 2 wk. Add growth factors and drugs when needed.

2.1.2.3. OPC Differentiation

1. Earle's balanced salt solution (EBSS; Invitrogen, cat. no. E2888).
2. 0.005% trypsin: add 0.1 mL 2.5% trypsin 2.5% (Invitrogen, cat. no. 25090-028) in 50 mL EBSS. Aliquot (5 mL) and store at –20°C.
3. Dulbecco's modified Eagle's medium (DMEM), high in glucose, pyruvate, pyridoxine (500 mL; Invitrogen, cat. no. 31966 021).
4. 100X penicillin-streptomicin-glutamine (PSG; Invitrogen, cat. no. 10 378-032).
5. Apo-transferrin (Sigma, cat. no. T1147).
6. Crystalline bovine serum albumin (Sigma, cat. no. A4161).
7. Progesterone stock solution (25 mg/mL): add 2.5 mg progesterone (Sigma, cat. no. P8783) to 100 µL ethanol (EtOH). Prepare a fresh stock each time Sato medium is to be made.
8. Putrescine (Sigma, cat. no. P5780).
9. Sodium selenite stock solution (0.4 mg/mL): add 4 mg sodium selenite (Sigma, cat. no. P8783) to 10 mL DMEM and 100 µL 0.1 *N* NaOH. Prepare a fresh stock each time Sato medium is to be made.

10. 100X *N*-acetyl-cysteine (40 m*M*): add 50 mg *N*-acetyl-cysteine (Sigma, cat. no. A9165) in 8.33 mL sterile water. Aliquot (200 µL) and keep at –20°C.
11. 1000X biotin (10 µg/mL): add 9 mg biotin (Sigma, cat. no. B4639) in 9 mL DMEM, then add 20 µL of this solution in 2 mL DMEM. Aliquot (25 µL) and keep at –20°C.
12. 5000X forskolin (25 m*M*): add 10 mg forskolin (Sigma, cat. no. F6886) in 975 µL dimethyl sulfoxide. Aliquot (50 µL) and keep at –20°C.
13. 1000X insulin (5 mg/mL): add 20 mg insulin (Sigma, cat. no. I6634) in 4 mL 0.01 *M* HCl. Sterilize with a 0.20-µ*M* filter. Aliquot (25 µL) and keep at –20°C.
14. 1000X NT3 (5 µg/mL): dilute 2 µg human NT3 (Peprotech, cat. no. 450-03) in 0.4 mL sterile PBS. Aliquot (25 µL) and keep at –80°C.
15. 1000X Shh (N-term) (0.3 mg/mL) (R&D systems, Oxon, UK; cat. no. 461-SH-025).
16. Sato 100X stock solution: combine 20 mL DMEM, 200 mg transferrin, 200 mg crystalline bovine serum albumin, 5 µL progesterone stock solution, 32 mg putrescine, and 200 µL sodium selenite stock solution. Aliquot (0.2 mL) and keep at –20°C.
17. OPC differentiation medium (Sato base): combine 20 mL DMEM, 200 µL PSG, 200 µL 100X Sato stock solution, 200 µL 100X *N*-acetyl-cysteine, 4 µL 5000X forskolin, 20 µL 1000X biotin, 20 µL 1000X insulin, 20 µL 1000X NT3. Keep this medium for 1–2 wk at 4°C. Add growth factor and drugs when needed.

2.1.2.4. Oligodendrocyte Differentiation

1. 1000X PDGF-AA (10 µg/mL): dilute 2 µg human PDGF (Peprotech, cat. no. 100-13A) in 0.2 mL filter-sterilized 10 m*M* acetic acid. Aliquot (20 µL) and store at –80°C.
2. 1000X T3 (triiodothyronine, thyroid hormone) (40 µg/mL): prepare a solution of 4 mg/mL T3 (Sigma, cat. no. T6397) in 0.1 *N* NaOH. Dilute this solution 100X in DMEM to make a 1000X solution. Aliquot (20 µL) and store at –80°C.

2.2. Analysis of Differentiation

2.2.1. Reverse Transcriptase Polymerase Chain Reaction

1. Thermal cycler.
2. Agarose gel apparatus and reagents.
3. Diethylpyrocarbonate-water.
4. RNeasy Mini Kit (Qiagen, West Sussex, UK; cat. no. 74103).
5. Oligo $dT_{12\text{-}18}$ (Invitrogen, cat. no. 18418-012).
6. Avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Southampton, UK; cat. no. M5101).
7. 10 m*M* dNTP mix (dATP, dCTP, dGTP, dTTP, each at 10 m*M*) (Promega, cat. no. C1141).
8. RNasin ribonuclease inhibitor (Promega, cat. no. N2511).
9. Polymerase chain reaction (PCR) 5′ and 3′ primers diluted to 3 µ*M* with sterile water.
10. Platinium *Taq* DNApolymerase high fidelity (Promega, cat. no. 11304-011).

2.2.2. Immunohistochemistry

1. PDL-coated 13-mm glass cover slips.
2. 4% paraformaldehyde: dilute 16% stock solution (Electron Microscopy Sciences RT 157-10) 1:4 in PBS and keep at 4°C for no more than 1 wk.
3. 10% goat serum: dilute goat serum (Invitrogen, cat. no. 16210072) 1:10 in PBS. Keep at 4°C for no more than 1 wk.
4. 0.1% Triton in 10% goat serum.
5. Monoclonal stage-specific embryonic antigen (SSEA) 1 antibody (diluted 1/5; Developmental Studies Hybridoma Bank, Iowa City, IA; cat. no. MC-480).

6. Monoclonal antirat nestin antibody (diluted 1/100, Pharmingen, San Diego, CA; cat. no. 556309).
7. Rabbit anti-NG2 chondroitin sulfate proteoglycan antibodies (diluted 1/50; Chemicon, cat. no. AB5320).
8. Monoclonal anti-GC antibody (supernatant, diluted 1/5) ***(16)***.
9. Conjugated secondary antibodies. Fluorescein isothiocyanate-coupled goat antimouse immunoglobulin or goat antirabbit immunoglobulin antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA; cat. no. 111095003 and cat. no. 115225003).
10. 500X Hoechst cat. no. 33342 (4 mg/mL): dilute 4 mg Hoechst (Sigma, cat. no. B2261) in 1 mL H_2O. Aliquot (100 µL) and store at –20°C away from light.
11. 95% EtOH-5% acetic acid (–20°C).
12. Citifluor mounting medium (Electron Microscopy Sciences, London, UK; cat. no. 17970).
13. Microscope slides.
14. Tissues.
15. Nail polish.
16. Microscopes. Cover slips were examined with a Zeiss Axioplan 2 fluorescence microscope. Nunclon multidishes were examined with a Leica DMIRB inverted fluorescence microscope.

3. Methods

To obtain a homogeneous population of neural precursor cells from which oligodendrocyte lineage cells originate, we used genetically engineered ES cells that allow the selection of such committed cells. Specifically, we used doubly targeted ES cells so that, after induction of differentiation, we could select negatively against residual ES cells and select positively for neural precursor cells. Genetic engineering of ES cells was done in A. Smith laboratory (Centre for Genome Research, Edinburgh, UK), and only the theoretical fundaments of it are briefly described here: to select against residual ES cells, a *hygromycin-thymidine-kinase* (*tk*) fusion gene was introduced into the *Oct4* locus ***(46)***. As *Oct4* is expressed in undifferentiated ES cells ***(47)***, such engineered ES cells should be eliminated by treatment with ganciclovir; to select for neural precursor cells, a β*geo* gene was introduced into the *Sox2* locus ***(45)***. As *Sox2* is specifically expressed in neural precursor cells ***(48)***, these cells should selectively survive treatment with G418 (*see* **Note 2**). Thus, by treating the doubly targeted ES cells with both ganciclovir and G418, undifferentiated ES cells should be selected against, and neural precursor cells should be selected for. Two independently derived parental ES cell lines were used for genetic engineering: CGR8 ***(49)*** and E14Tg2a ***(50)***. Both lines support neural and oligodendrocyte differentiation and should be available on request from the Smith lab.

3.1. Tissue Culture

3.1.1. Maintenance of ES Cells

CGR8 and E14Tg2a ES cells can be grown on gelatin-coated TC dishes in GMEM supplemented with 10% serum and LIF. ES cells must be passaged every other day, and they should never reach more than 90% confluence to obtain good neural differentiation afterward. Serum can affect ES cell growth and differentiation, so it should be batch tested (*see* **Note 1**). Mycoplasma contamination can also have profound effects

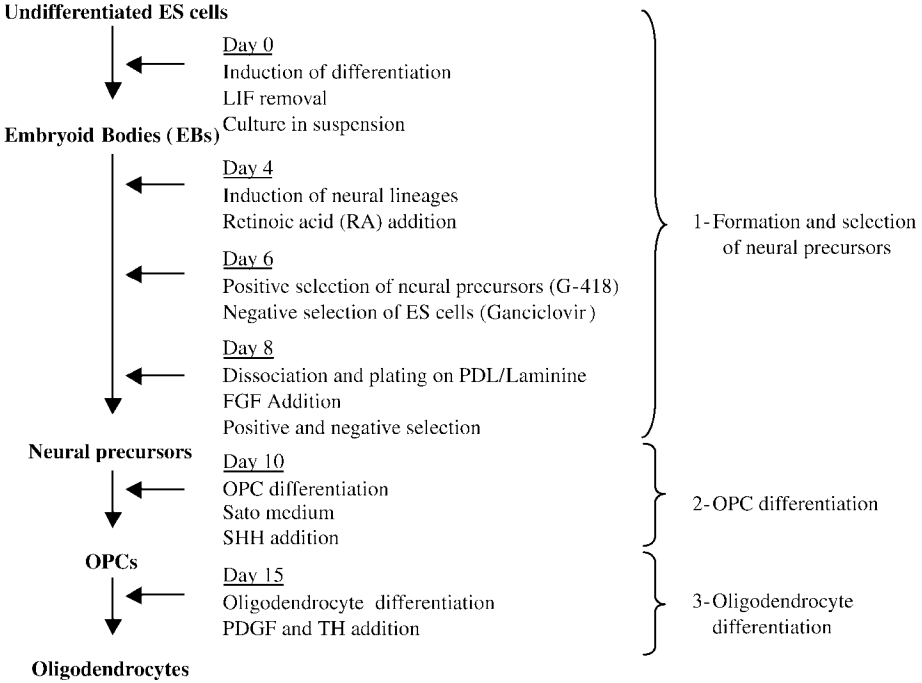

Fig. 1. Schematic view of the differentiation protocol.

on ES cell growth and differentiation, so it is recommended that cells are tested routinely for mycoplasma (*see* **Note 3**).

To subculture ES cells:

1. Prepare a 0.1% gelatin solution by diluting 2% gelatin in PBS, 37°C. Add 5 mL of this solution to a 25-cm^2 flask and incubate for about 20 min.
2. During this time, passage the cells. Gently remove medium and wash with 10 mL PBS-EDTA. Remove PBS and add 1.5 mL trypsin. Return flask to incubator for 5 min.
3. Gently agitate the flask. When the cells are floating, neutralize trypsin with 8.5 mL neutralization medium. Pipet up and down several times to make a single-cell suspension and transfer the cells into a universal tube.
4. Collect the cells by centrifugation at 700*g* for 5 min.
5. Resuspend in 10 mL ES cell medium plus LIF (10^3 U/mL).
6. Count cells (usually 10^7 cells can be obtained from a 25-cm^2 flask).
7. Remove gelatin from the flask and plate 1.5×10^6 cells/flask in 5 mL ES cell medium plus LIF.

3.1.2. Differentiation of ES Cells Into the Oligodendrocyte Lineage

Our differentiation protocol consists of three distinct stages, outlined in **Fig. 1**.

Stage 1: Formation and selection of neural precursors. We have used a standard way to induce ES cells to differentiate, which is to allow them to aggregate in vitro in the absence

of LIF to form EBs. Treatment with RA at d 4 encourages the cells in the EBs to differentiate into neural cells, which can then be selected by the addition of G418 at d 6. Furthermore, undifferentiated ES cells are removed by adding ganciclovir to the medium. At d 8, a good proportion of neural precursors have formed within the EBs, and they are dissociated and encouraged to develop further by plating out on a PDL-laminin substrate in the presence of FGF-2, which has been shown to be important for neural precursors proliferation in vitro *(51)* and in vivo *(52)*. Positive and negative selections are maintained for another 2 d to enrich for neural precursors.

Stage 2: OPC differentiation. By d 10 of differentiation, more than 85% of ES cell-derived cells are neural precursors, and undifferentiated ES cells have been eliminated. The purified population of neural precursors can then be induced to differentiate through the oligodendrocyte lineage. This is achieved by culturing the cells in a serum-free, modified Bottenstein-Sato medium known to be permissive for oligodendrocyte development in vitro *(53)* in the presence of Shh, which has been shown to promote the development of OPCs from neural precursors in vitro and in vivo *(54–57)*.

Stage 3: Oligodendrocyte differentiation. By d 15 of differentiation, OPCs have formed, and they can be induce to differentiate by PDGF and thyroid hormone, which have been shown to promote OPC differentiation in cultures of purified rat OPCs *(23–26)*.

3.1.2.1. Coating of the Plates

At some point during the first 8 d of differentiation, make sure to coat Falcon 25-cm^2 flasks and Nunc four-well plates with PDL:

1. Prepare a 10-µg/mL PDL solution by diluting 1 mg/mL 100X PDL in sterile water. This diluted solution can be kept for up to 2 wk at 4°C.
2. Cover the bottom of the plates with this solution and leave under the hood for 5–15 h.
3. Remove PDL solution and wash the plates three times with sterile water.
4. Let the plates dry under the hood (1–12 h).
5. Use the plates immediately or store them for up to 2 wk at room temperature.

3.1.2.2. Formation and Selection of Neural Precursors

1. At 1 d before differentiation, passage the cells as described in **Subheading 3.1.1.** Replate the cells at 3–5 × 10^6 cells/flask so that they reach 90% confluency on the day differentiation is started.
2. On d 0 of differentiation, trypsinize the cells as in **Subheading 3.1.1.** At **step 3**, add 10 mL neutralization medium and resuspend cell pellet by gently shaking the tube (no extensive pipetting). At this stage, it is recommended to leave small aggregates of two to five cells instead of single cells as it seems to promote further differentiation.
3. Collect cells by centrifugation and gently resuspend them (as in **step 2**) in 5 mL ES cell medium (without LIF). Count cells (a single-cell suspension can be obtained by pipetting a small aliquot with a 200-µL pipetboy).
4. Put 7 × 10^6 cells in a 10-cm bacterial dish and complete to 10 mL with ES cell medium.
5. Check for small cell aggregates under the microscope. If there are too many single cells, then repeat centrifugation and gently resuspend the pellet as in **step 2**.
6. Return cells to the incubator and check for the formation of EBs every day under the microscope (they should be visible from d 1) (*see* **Note 4**).
7. Change ES cell medium every other day. To change the medium, gently transfer EBs into a universal container with a 10-mL plastic pipet. When EBs have settled (5 min),

gently remove supernatant by aspiration. Add fresh medium and transfer EBs into a new bacterial dish.
8. On d 4, when changing the medium, add 10^{-6} *M* RA (2 µL 5000X stock in 10 mL ES cell medium).
9. On d 6, when changing the medium, replace RA-containing ES cell medium by neural differentiation medium (F12-N2-NB-B27). Start selection of neural precursors by adding 100 µg/mL G-418 (5 µL 2000X stock in 10 mL medium).
10. On d 8, dissociate EBs and plate them on coated 25-cm^2 flasks.

3.1.2.3. Coating PDL-Coated 25-cm^2 Flasks With Laminin

1. Dilute 1 mg/mL laminin 1/100 in sterile PBS.
2. Put 5 mL of this solution in a 25-cm^2 flask precoated with PDL.
3. Put the flasks in the incubator while dissociating EBs (approx 1 h).
4. Remove laminin just before plating the cells (do not rinse).

3.1.2.4. Dissociation of EBs

1. Wash EBs with 10 mL PBS (let EBs settle and then remove PBS).
2. Add 0.5 mL warm 4X trypsin and incubate at 37°C in a water bath for 5–10 min. Carefully check for EB dissociation (*see* **Note 5**).
3. Neutralize trypsin with 10 mL neutralization medium and dissociate EBs into single cells by gently pipetting up and down with a 10-mL pipet.
4. Collect cells by centrifugation at 700*g* for 5 min.
5. Wash once with 10 mL DMEM to remove all traces of serum.
6. Collect cells by centrifugation at 700*g* for 5 min.
7. Resuspend cells in 5 mL neural differentiation medium by pipetting up and down three or four times with a 5-mL glass pipet.
8. Count cells. Usually, 5×10^6 to 10^7 cells can be obtained from one 10-cm bacterial dish. Flask 3×10^6 cells per 25-cm^2 flask (precoated with PDL and laminin) in 5 mL differentiation medium.
9. Add 20 µg/mL FGF-2 (5 µL 1000X stock) to promote neural precursor proliferation, 100 µg/mL G-418 (2.5 µL 2000X stock) to select for neural precursors and 2.5 µ*M* ganciclovir (5 µL 1000X stock) to select against any residual ES cells.
10. Maintain cells in this medium for 2 d more to favor the expansion of neural precursors.

This procedure results in progressive enrichment in neural precursors together with a decrease in undifferentiated ES cells (**Fig. 2**). By d 10, it reproducibly generates a cell population containing more than 85% nestin-expressing neural precursors.

3.1.2.5. OPC Differentiation

On d 10 of differentiation, selection can be stopped, and OPCs can be induced to differentiate from the homogeneous population of neural precursors. OPCs can be identified from d 15 by staining for NG2 (**Fig. 3**).

1. Coat PDL-coated Nunc four-well plates with laminin as in **Subheading 3.1.2.3.** and prepare OPC differentiation medium.
2. To passage neural precursors, gently wash the cells with warm EBSS and add 1 mL trypsin 0.005%. Return to the incubator for 1–5 min (check for floating cells).

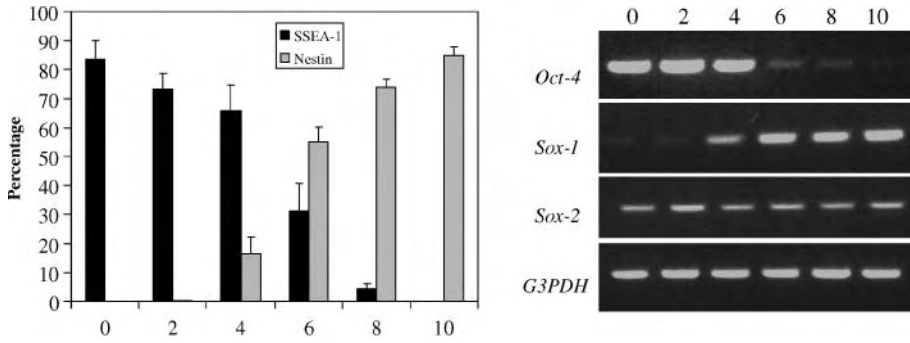

Fig. 2. Development and selection of neural precursors from engineered embryonic stem (ES) cells. Engineered ES cells were treated and selected as described in **Fig. 1** to enrich for neural precursors and to eliminate residual undifferentiated ES cells. At various times after the start of differentiation, the cells were dissociated and either stained for stage-specific embryonic antigen or nestin by immunohistochemistry (**left**) or processed for reverse transcriptase polymerase chain reaction analysis using *Oct4*, *Sox1*, *Sox2*, or *G3PDH* probes (**right**).

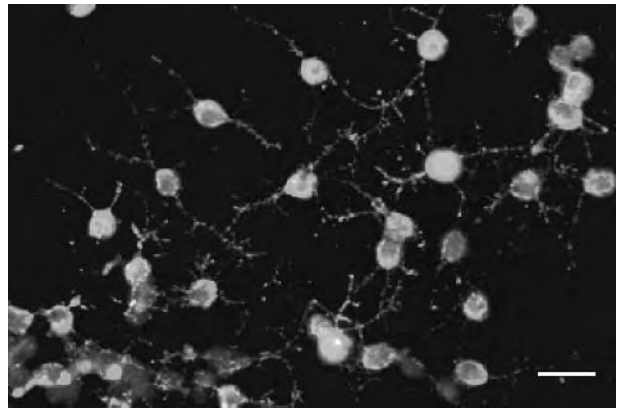

Fig. 3. Development of OPCs from embryonic stem cell-derived neural precursors. At d 15 of differentiation, the cells were stained with anti-NG2 antibody to identify oligodendrocyte precursor cells and bisbenzimide to identify cell nuclei.

3. When the first cells are floating, add 9 mL neutralization medium and gently pipet up and down. Collect the cells by centrifugation at 700*g* for 5 min.
4. Wash the cells with 10 mL DMEM to remove all trace of serum.
5. Collect cells by centrifugation and gently resuspend the cells in 5 mL Sato medium.
6. Count the cells and plate them in four-well plates coated with PDL and laminin. Put 5×10^4 cells/well (0.5 mL/well) in OPC differentiation medium containing 20 ng/mL FGF-2 and 0.3 µg/mL Shh.
7. Change half of the medium every other day for 5 d (until d 15 of differentiation). If the cells become confluent during this time-course, then passage them as described in **steps 2–6**.

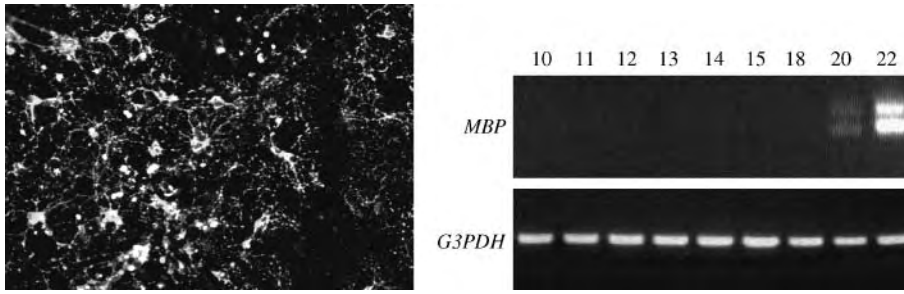

Fig. 4. Development of oligodendrocytes from embryonic stem cell-derived oligodendrocyte precursor cells. At d 22 of differentiation, the cells were stained with anti-galactocerebroside antibody to identify oligodendrocytes and bisbenzimide to identify cell nuclei (**left**). At various times after the start of differentiation, the cells were processed for reverse transcriptase polymerase chain reaction analysis using myelin basic protein or G3PDH probes (**right**).

3.1.2.6. Oligodendrocyte Differentiation

On d 15 of differentiation, OPCs have formed and can be induced to differentiate. Oligodendrocytes can be identified from d 22 by GC staining or expression of myelin basic protein (MBP) mRNA (**Fig. 4**).

1. Replace medium with Sato medium containing 10 ng/mL PDGF-AA and 40 ng/mL T3.
2. Change half of the medium every other day for 7 d (until d 22 of differentiation).

3.2. Analysis of Differentiation

A variety of markers can be used to follow the fate of ES cells and to help identify neural precursors, OPCs, and oligodendrocytes as outlined in **Fig. 5**. We have used reverse transcriptase (RT) PCR analysis to detect *Oct4, Sox1*, *Sox2,* and *MBP* mRNAs and immunohistochemistry to detect SSEA-1, nestin, NG2, and GC antigens (*see* **Note 6**). We scored cells as positive in immunohistochemical assays only if they also had the characteristic morphology of OPCs and oligodendrocytes ***(58)***.

3.2.1. Reverse Transcriptase Polymerase Chain Reaction

The sequences of the primers that we used to detect *Oct4, Sox1*, *Sox2,* and *MBP* mRNAs are indicated in **Table 1**.

3.2.1.1. RNA Extraction

If RNA is to be extracted from EBs, then proceed to **step 1**. If RNA is to be extracted from neural precursors, OPCs, or oligodendrocytes, then trypsinize cells as in **Subheading 3.1.2.3., steps 2–6**. After neutralization of the trypsin with serum-containing medium,

1. Harvest cells by centrifugation at 700*g* for 5 min.
2. Remove medium and gently wash the cell pellet with 5 mL warm PBS.
3. Facultative: count cells and keep a record of cell numbers for further RNA extraction.
4. Collect cells by centrifugation at 700*g* for 5 min.

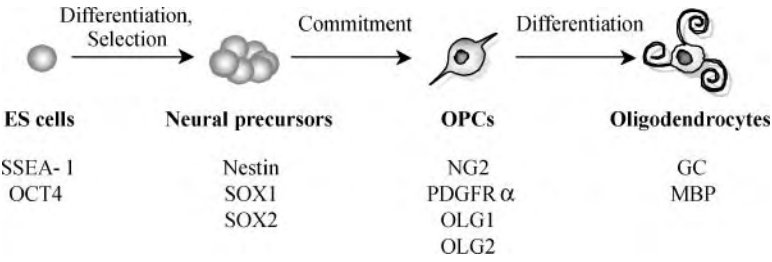

Fig. 5. Markers used to identify embryonic stem cells, neural precursors, and oligodendrocyte lineage cells.

Table 1
Primer Sequences

Gene	Forward primer	Reverse primer
Oct4	5′-CTG CTG AAG CAG AAG AGG ATC AC-3′	5′-TGG TTC TGT AAC CGG CGC CAG AAG-3′
Sox1	5′-TTA CTT CCC GCC AGC TCT TC-3′	5′-TGA TGC ATT TTG GGG GTA TCT CTC-3′
Sox2	5′-AAC ATG ATG GAG ACG GAG CTG AAG C-3′	5′-TAC GCG CAC ATG AAC GGC TGG AG-3′
MBP	5′-AAG TAC TTG GCC ACA GCA AG-3′	5′-CAG AGC GGC TGTCTC TTC-3′
G3PDH	5′-ACC ACA GTC CAT GCC ATC AC-3′	5′-TCC ACC ACC CTG TTGCTG TA-3′

5. Remove supernatant, add 1 mL fresh PBS, and transfer the cells to an Eppendorf tube.
6. Centrifuge at maximum speed for 1 min.
7. Remove supernatant and snap-freeze the cell pellet on dry ice (*see* **Note 7**).
8. Extract RNA using the RNeasy Mini Kit from Qiagen following the manufacturer's instructions for isolation of cytoplasmic RNA from animal cells.
9. At the last step, elute RNA in 30 µL RNase-free water.
10. Find the optical density (OD) at A_{260} and A_{280}. Determine the concentration of the RNA sample (*see* **Note 8**).

3.2.1.2. Reverse Transcription

1. In a PCR tube, add 1 µg RNA diluted in water to a final volume of 4 µL and 1 µL Oligo $dT_{12\text{-}18}$ (Gibco BRL).
2. Heat at 70°C for 5 min.
3. Cool on ice for 5 min.
4. Prepare the RT mix (*see* **Note 9**): 5X AMV reverse transcriptase buffer, 2.5 µL 10 m*M* dNTP mix, 1 µL RNAsin, AMV-RT, and 8.5 µL H_2O.
5. Add the RT mix to the RNA and vortex briefly.
6. Incubate at 42°C for 60 min.

Table 2
Annealing Temperature and Number of Cycles for PCR Amplification

Gene	Annealing temperature	Number of PCR cycles
OCT4	62°C	35
SOX1	62°C	25
SOX2	62°C	35
MBP	53°C	26
G3PDH	60°C	25

7. Incubate at 95°C for 10 min.
8. Cool on ice for 5 min.

3.2.1.3. Polymerase Chain Reaction

1. Prepare the PCR mix (25 µL final volume): 1 µL template complementary DNA, 2.5 µL 10X high-fidelity PCR buffer, 0.5 µL 25 m*M* $MgSO_4$, 0.5 µL 10 m*M* dNTP mix, 2.5 µL 3 µ*M* 5′ primer, 2.5 µL 3 µ*M* 3′ primer, 0.2 µL *Taq* DNA polymerase high fidelity, and 15.8 µL H_2O.
2. Centrifuge the tubes briefly to collect the contents.
3. Incubate tubes in a thermal cycler at 94°C for 2 min.
4. Perform 25–35 cycles of PCR amplification as follows: the number of cycles and the annealing temperature depends on the primers used (*see* **Table 2**): 94°C for 30 s, X°C for 30 s, 72°C for 90 s.
5. Incubate at 72°C for 5 min.
6. Maintain the reaction at 4°C.
7. Analyze the products by agarose gel electrophoresis and visualize by ethidium bromide staining. Use appropriate molecular weight standards.

3.2.2. Immunohistochemistry

Before starting the staining procedure, cells must be plated at low density (10^5 cells/cover slip) on PDL-coated cover slips. They are then left on the cover slips for a minimum of 2 h before they can be fixed and stained. If staining is to be performed on EBs, **then** they first have to be trypsinized as indicated in **Subheading 3.1.2.4.** and then seeded on cover slips. If staining is to be performed on dissociated neural precursors, OPCs, or oligodendrocytes, **then** cells can be grown directly on cover slips or trypsinized and put on cover slips just before staining, as for EBs.

3.2.2.1. PDL Coating of Glass Cover Slips

1. Wash cover slips in 70% EtOH for 1 h (with agitation).
2. Replace with fresh EtOH and incubate overnight with agitation. Replace with fresh EtOH and keep until use.
3. When ready to use, wash the cover slips with sterile water about five or six times (rapid washes) and put the cover slips in four-well plates.
4. Proceed with coating of cover slips. Coat with PDL as described in **Subheading 3.1.2.1.**

3.2.2.2. Staining Procedure

1. Gently wash the cells with warm PBS.
2. Fix in 4% paraformaldehyde for 5 min.
3. Wash twice with PBS.
4. Add 10% goat serum and incubate for 30 min to block nonspecific staining.
5. When needed (nuclear or cytoplasmic antigen), permeabilize membranes by incubating the cells in 0.1% Triton (diluted in goat serum) for 30 min. For membrane antigens, directly proceed to **step 7**.
6. Wash twice in PBS.
7. Incubate with primary antibody (diluted in 10% goat serum) for 60 min.
8. Wash four times in PBS.
9. Incubate with conjugated secondary antibody (diluted 1/100 in 10% goat serum) and Hoechst 33342 (diluted 1/500, for coloration of the nucleus) for 60 min.
10. Wash four times in PBS.
11. Postfix the cells with 95%EtOH-5% acetic acid (–20°C) for 30 s (*see* **Note 10**).
12. Wash twice with PBS.
13. Mount cover slips on a slide: add a very small drop of Citifluor on the slide with a 200-µL tip; gently pour over the cover slip and absorb the excess of liquid with a tissue. Seal with nail polish. Wait about 45 min before looking at the slide under the microscope.

4. Notes

1. The ability of serum to support growth of pluripotent ES cells is crucial. Every time new serum is to be ordered, several lots should be tested, and suitable batches must be ordered in large quantities to avoid variability. As an indication, we usually consider a serum suitable if it allows sustained ES cell growth (10^7 cells must be obtained out of 1.5×10^6 cells after 48 h for at least three passages), and it is not toxic at a 30% concentration. A number of suppliers now sell sera that have already been tested for supporting ES cell growth.
2. Undifferentiated ES cells also express *Sox2* and would be expected therefore to survive G418; they should be eliminated by ganciclovir.
3. Several methods and kits are now available to test for Mycoplasma contamination. We have been successfully using kits from ATCC, Venorgem, and Gibco-BRL.
4. To give nice neural precursors, EBs should be compact with a round, three-dimensional shape, dark in the middle with a large surrounding light band, or completely light. When EBs have an irregular shape and a smooth appearance, are entirely dark (meaning that there are many dead cells), or stick to the bottom of the dish, it is usually a bad prognostic for neural differentiation.
5. EB dissociation is an important step. Overtrypsinization may result in cell death; undertrypsinization may keep EBs as cell aggregates that may not proceed correctly with neural differentiation. Therefore, it is recommended to carefully check for EB dissociation during trypsinization—when ready, the solution should become cloudy—and stop it when necessary. If too many cell aggregates are still present after dissociation, then repeat trypsinization.
6. Other proteins that are characteristic of oligodendrocytes and their precursors are expressed earlier than NG2 and may therefore be used to identify the earliest stages of oligodendrocyte lineage specification in ES cell-derived cultures. These include the *Olig1* and *Olig2* basic helix-loop-helix gene regulatory proteins ***(11,12)***, as well as PDGFR-α, which can be detected by *in situ* hybridization or RT-PCR ***(46)***.
7. Cell extracts may be stored at –80°C until RNA extraction.

8. The A_{260}/A_{280} ratio should be between 1.6 and 2. To determine RNA concentration, use the following equation: [ ] µg/µL = [$A_{260} \times 40 \times$ Dilution factor]/1000.
9. As genomic DNA has not been removed from RNA samples, always include a control for which RT is omitted and replaced by water.
10. This allows the cells to stick better on the cover slip, and it amplifies the signal.

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3

Derivation and Characterization of Neural Cells From Embryonic Stem Cells Using Nestin Enhancer

Nibedita Lenka

Summary

The embryonic stem (ES) cells derived from the inner cell mass of the blastula stage embryo bear the complete repertoire of the complex organizational blueprint of an organism. These fascinating cells are bestowed with pluripotent characteristics and can be directed to differentiate into various lineages in vitro. Hence, these cells are being explored as an ideal in vitro model in gaining insight into early developmental events. Using the ES cell system, we have tried to investigate the early neurogenic proceedings. We have taken advantage of nestin enhancer-mediated cell trapping using the live reporter-based system. We monitored live the ES cell differentiation into neural lineage by following the enhanced green fluorescent protein expression profile in a number of stable ES cell clones generated by us in which the enhanced green fluorescent protein expression was regulated by the nestin enhancer. This strategy has helped us in both qualitative and quantitative detection and characterization of neural progenitor population and the differentiated progenies.

Key Words: ES cells; glia; live reporter; nestin; neural stem cells; neurogenesis; neuron.

1. Introduction

The intricacy of early embryonic development involves a complex organizational interplay among factors regulating cell proliferation, differentiation, and apoptosis. The decoding of this blueprint needs a comprehensive investigation of the key players involved in the cell fate decision machinery and understanding their precise role. In this context, embryonic stem (ES) cells derived from the inner cell mass of the blastula stage embryo can be explored as a tangible in vitro model system to understand these developmental complexities.

Neurogenesis, as one of the most complex events during organogenesis, involves an intricate signaling and cellular interaction cascade to generate a functional neural network. To investigate the early neurogenic events, we opted for pluripotent ES cells that are indeed capable of recapitulating in vivo events in a relatively precise manner ***(1)***. However, the pluripotency leads to the generation of various cell types from ES cells in vitro, thus making it ambiguous in contemplating the exact phenotypes of cells by microscopic

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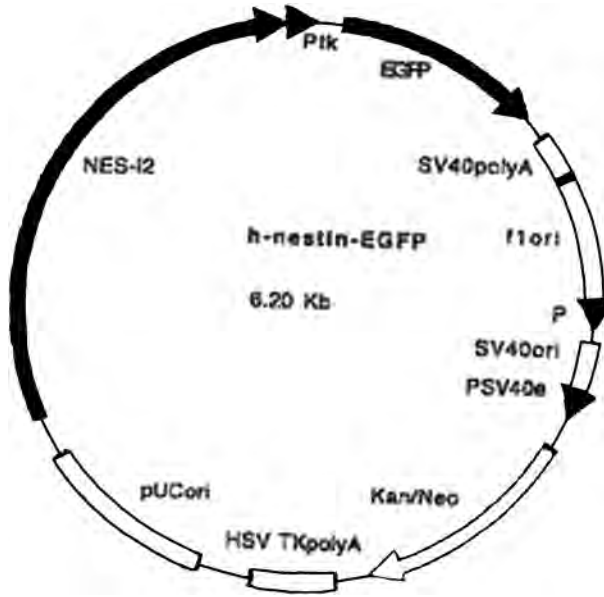

Fig. 1. The DNA construct (h-nestin-EGFP) used for transfecting the murine embryonic stem cell line D3 and obtaining the stable clones for nestin green fluorescent protein.

observations. In this regard, the tissue-specific, promoter-mediated trapping of desired cell types comes into being as an efficient approach in the identification, isolation, and functional characterization of lineage-specific populations in the heterogeneous cell mass of the ES cell system *(2,3)*. In this chapter, we demonstrate the targeting, specification, and characterization of neural progenies from murine ES cells using the neural stem cell marker nestin.

Nestin, the name spun from neuroepithelial stem cells, is universally proclaimed as the marker for neural stem cells. It was initially discovered as a member of the intermediate filament family protein belonging to class VI *(4)*. The gene structure reveals it to have four exons and three introns. Interestingly the 5′ upstream promoter region of nestin serves merely as basal transcriptional machinery; the introns play a decisive role in imparting tissue specificity *(5)*. Earlier studies on the nestin gene using transgenic mice indeed demonstrated the first intron rendering specificity to skeletal muscle-specific nestin expression, with the second intron having the necessary *cis*-acting enhancer motifs that drive reporter gene expression in a neuron-specific manner in the developing central nervous system (CNS) *(5–9)*. Nestin is expressed in a majority of mitotically active CNS and peripheral nervous system progenitors *(4,10–12)*. However, the expression is downregulated on differentiation *(5,6)* and has been reported to reappear on injury *(10,13–15)*. In fact, nestin represents a much broader spectrum, multipotent neural lineage marker as the nestin-expressing cells are capable of giving rise to all the cell types of the nervous system, that is, the neurons, astroglia, and oligodendroglia *(3,16–18)*. Thus, the cells expressing nestin show all the characteristic features of stem cells, such as multipotency, self-renewal, and regeneration.

Hence, we used nestin as an efficient candidate marker gene to explore early neurogenic proceedings from ES cells in vitro. Moreover, we chose nestin-mediated targeting in view of generating and demarcating both neurons and glia in a single system, that is,

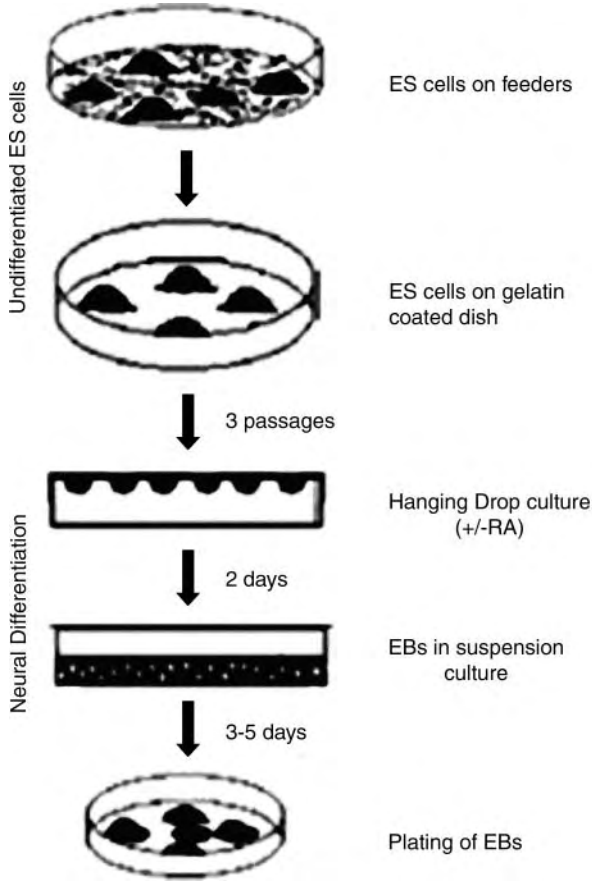

Fig. 2. Scheme showing the maintenance of embryonic stem cells and differentiation into the neural lineage in vitro.

the ES cell model for deciphering the underlying mechanism of neurogenesis and gliogenesis besides unraveling the molecular determinants differentiating the events from a same set of stem/progenitors.

We have taken the human nestin intron II enhancer fused to the thymidine kinase (tk) basal promoter (for transcription initiation) and the enhanced green fluorescent protein (EGFP) reporter (**Fig. 1**) that, on introduction into ES cells, regulates the expression of live reporter EGFP in a neural-specific manner. This strategy has helped us identify, quantify, and functionally characterize the ES cell-derived, development-dependent, proliferating neuronal progenitors as well as the differentiating neurons based on the nestin intron II-driven EGFP expression profile. We have successfully established a number of stable G418-resistant nestin green fluorescent protein (GFP) transgenic ES cell clones using the murine ES cell line D3.

Several of these selected clones were differentiated into various neural phenotypes following the in vitro differentiation protocol (**Fig. 2**) and simultaneously monitored for EGFP expression. Surprisingly, 96% of the G418-resistant nestin ES cell clones were

observed to be EGFP positive even at the undifferentiated state with a heterogeneous distribution pattern of EGFP expression (**Fig. 3A**). With the help of immunocytochemistry, we specified the EGFP-expressing cells as nestin positive (**Fig. 3B**,**C**), indicating the reliability/authenticity of our approach. The validation of early onset of endogenous nestin and concomitant EGFP expression was also confirmed by reverse transcriptase polymerase chain reaction, indirectly implying the nestin transcription as active even in undifferentiated ES cells prior to any lineage commitment ***(3)***. As expected, however, during ES cell differentiation EGFP expression remained confined to the neural lineage. The immunocytochemical characterizations of nestin GFP clones at various time-points during differentiation exemplified the endogenous nestin expression profile from ES cells in vitro, corroborating that of the EGFP expression pattern.

Although the neural stem cells/progenitors and early neurons showed bright EGFP expression (**Fig. 3D**,**E**) and nestin immunoreactivity (nestin$^+$/EGFP$^+$; **Fig. 3G**), the more mature ones (MAP2$^+$; **Fig. 3H**) that remained in close association with the EGFP$^+$ neural progenitors were either negative or very weakly positive for EGFP. Similarly, the mature oligodendroglia (**Fig. 3K**) and astroglia were EGFP$^-$; some of the astroglia that retained the potential to divide ***(3)*** also retained weak EGFP (GFAP$^+$; **Fig. 3J**). This is in line with the endogenous nestin expression pattern, for which its expression is pronounced in mitotically active (i.e., actively dividing) cells, showing downregulation during differentiation. Using a number of antigenic markers belonging to the neural lineage, we could characterize not only the neural stem cells/progenitors, but also the differentiated neurons and glial cells. Even various subtypes of neurons (dopaminergic; **Fig. 3L**,**M** and serotonergic ones) were also identified in our culture system.

The qualitative pattern of neural differentiation from ES cells is quite asynchronous. Hence, to discern the optimum time window for the maximum numbers of neural progenitors, we attempted a quantitative estimation of the same by flow cytometric detection of EGFP expression. Indeed, it showed the window to lie between 4 and 10 d postplating (***3***; **Fig. 4**). This information not only provided insight into the time- and development-dependent neural progenitor induction, but also gave impetus in designing a further strategy for investigating real-time monitoring of neuro- (**Fig. 5**) and gliogenesis from the progenitor population by time-lapse recordings ***(3)*** of the events from 1-wk postplated EBs. Moreover, the same time window was also selected for transplantation experiments to verify the efficacy of neural progenitors in cell replacement therapy ***(18)***. The patch-clamp recordings at various time-points during differentiation also helped us in the functional characterization of these cells as well as the differentiating neurons ***(3)***. Thus, nestin enhancer-mediated cell trapping helped us gain insight into both qualitative and quantitative neurogenesis from ES cells in vitro and provided the platform for exploring the potential of neural stem cells in transplantation therapies.

2. Materials

2.1. Vectors

1. The Nes 1852 tk/lacZ DNA construct, encompassing the intron II of human nestin, heterologous tk basal promoter, and lacZ reporter in the pBS (blue script) backbone was a kind gift of Dr. Urban Lendahl, Sweden ***(6)***.
2. pEGFP1 promoterless enhanced EGFP reporter construct (Clontech, Palo Alto, CA; cat. no. 6086-1).

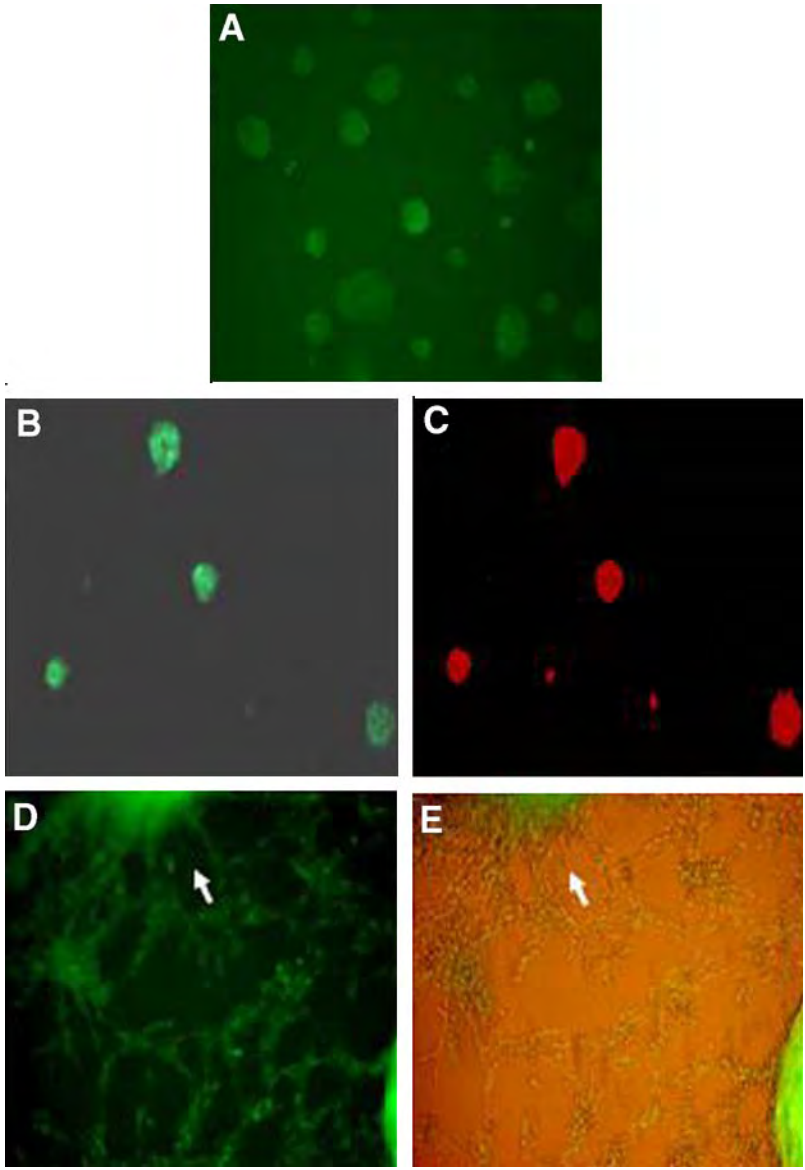

Fig. 3.

2.2. Cells

1. Mouse ES cells line D3 (ATCC, Manassas, VA; cat. no. CRL-1934).
2. STO fibroblast (ATCC, cat. no. CRL-1503).
3. STO-SNL2 (neomycin-resistant STO fibroblast) (ATCC, cat. no. CRL-2225).
4. Mouse embryonic fibroblasts (MEFs) derived from E12.5- to E14.5-d-old mouse embryos.

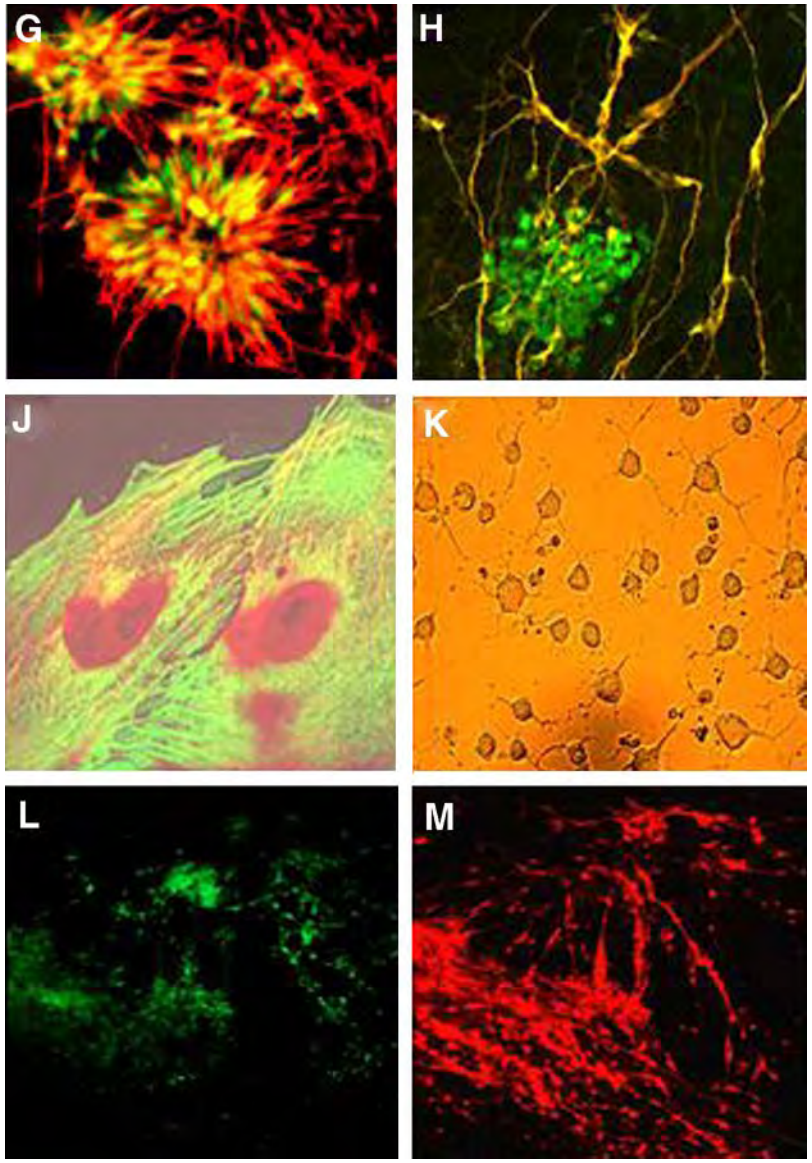

Fig. 3. EGFP expression and immunocytochemical characterization of nestin green fluorescent protein (GFP) embryonic stem (ES) cell clones. (**A**) The human nestin intron II-driven EGFP expression in undifferentiated ES cells during propagation on mitotically inactivated fibroblast feeders. (**B**) The enhanced (EGFP) expression corresponds to the endogenous nestin expression (**C**) in undifferentiated ES cells propagated on gelatin-coated glass cover slips. (**D**,**E**) Nestin GFP ES cell clones during differentiation: the embryoid bodies (EBs) following retinoic acid exposure during hanging drop preparation and subsequent plating show differentiation into neural cells (7 d postplating). The neural progenitors and early neural derivatives show EGFP expression

2.3. Reagents and Kits for Cloning (19)

1. Luria-Bertani (LB) medium: for 1 L, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 800 mL H_2O, adjust to pH 7.4, and autoclave after making the final volume. For LB plating medium, add 1.5 g/L bacto-agar to the liquid medium, autoclave, and when the medium temperature decreases to 50–56°C, pour onto 100-mm plates with/without desired antibiotics (30 µg/mL kanamycin, 100 µg/mL ampicillin), and keep the lid half open for solidification. The plates can be stored for 1–2 wk at 4°C for future use without any loss of antibiotic effect.
2. Competent cells (DH5/XL1 blue).
3. Solution I: prepare 1 *M* stock solution of D-glucose (18 g in 100 mL) in water, filter-sterilize, and store the solution at 4°C. Prepare 1 *M* and 0.5 *M* stock solutions of Tris (12.1 g Tris-base in 100 mL H_2O) and ethylenediaminetetraacetic acid (EDTA; 18.6 g in 100 mL H_2O), respectively, adjust to pH 8.0, autoclave, and store at room temperature ***(19)***. For working solution, mix the desired volumes from the stocks (for 100 mL, 5 mL D-glucose, 2.5 mL Tris, and 2 mL EDTA) to obtain the final concentration as 50 m*M* D-glucose, 25 m*M* Tris, and 10 m*M* EDTA.
4. Solution II: prepare the 10X stocks separately for NaOH (2 *N*) and sodium dodecyl sulfate (10%). Store at room temperature. Prior to use, mix both to obtain the working solution as 0.2 *N* NaOH and 1% sodium dodecyl sulfate.
5. Solution III: for 100 mL, mix 60 mL 3 *M* potassium acetate (29.4 g in 100 mL H_2O) with 11.5 mL glacial acetic acid and make up the final volume with water ***(19)***. Store the solution at 4°C.
6. Isopropanol.
7. Ribonuclease (New England Biolabs [NEB], Beverly, MA; cat. no. MQ243S).
8. Buffer-saturated phenol (Sigma, St. Louis, MO; cat. no. P4557).
9. Chloroform.
10. Sodium acetate.
11. Ethyl alcohol.
12. TE (pH 8.0): for 100 mL, mix 1 mL 1 *M* Tris at pH 8.0 with 0.2 mL 0.5 *M* EDTA at pH 8.0 and make the final volume with water to obtain 10 m*M* Tris and 1 m*M* EDTA solution.

Fig. 3. *(Continued)* (**D**, fluorescence alone; **E**, the same in dual-mode bright field and fluorescence combined), and the arrow indicating the central part of the EB from which the neural differentiation extends in a centrifugal fashion. (**G**) A complete overlap (yellow) between the EGFP-expressing cells (green) and the endogenous nestin (red) in the 7-d postplated EBs discerned by immunocytochemistry validates the nestin-specific EGFP expression in the nestin GFP clones. As would be expected, the nestin-expressing non-EGFP cells (red alone) specify the nonneural population generated from differentiating EBs. (**H**) The neuronal identity of the EB-derived cells discerned by immunocytochemistry in which the differentiating neurons are showing MAP2 immunoreactivity, a marker for differentiated neurons. The MAP2-positive cells are $EGFP^-$, indicating the downregulation of endogenous nestin expression in these cells; however, they remain in close association with $EGFP^+$ neural progenitors. (**J**) The EBs differentiating into astroglia lineage show GFAP immunoreactivity, some of which still retain weak EGFP expression, whereas the cells differentiating into oligodendroglialike cells (**K**) are $EGFP^-$. (**L**,**M**) The differentiation of nestin GFP clones into dopaminergic neurons. In 2-wk-old postplated EBs a number of dopaminergic neurons (tyrosine hydroxylase immunoreactive, (**M**) are seen in the close vicinity of EGFP-expressing (**L**) neural progenitors/early differentiating neurons. (Please *see* companion CD for **Fig. 3** in color.)

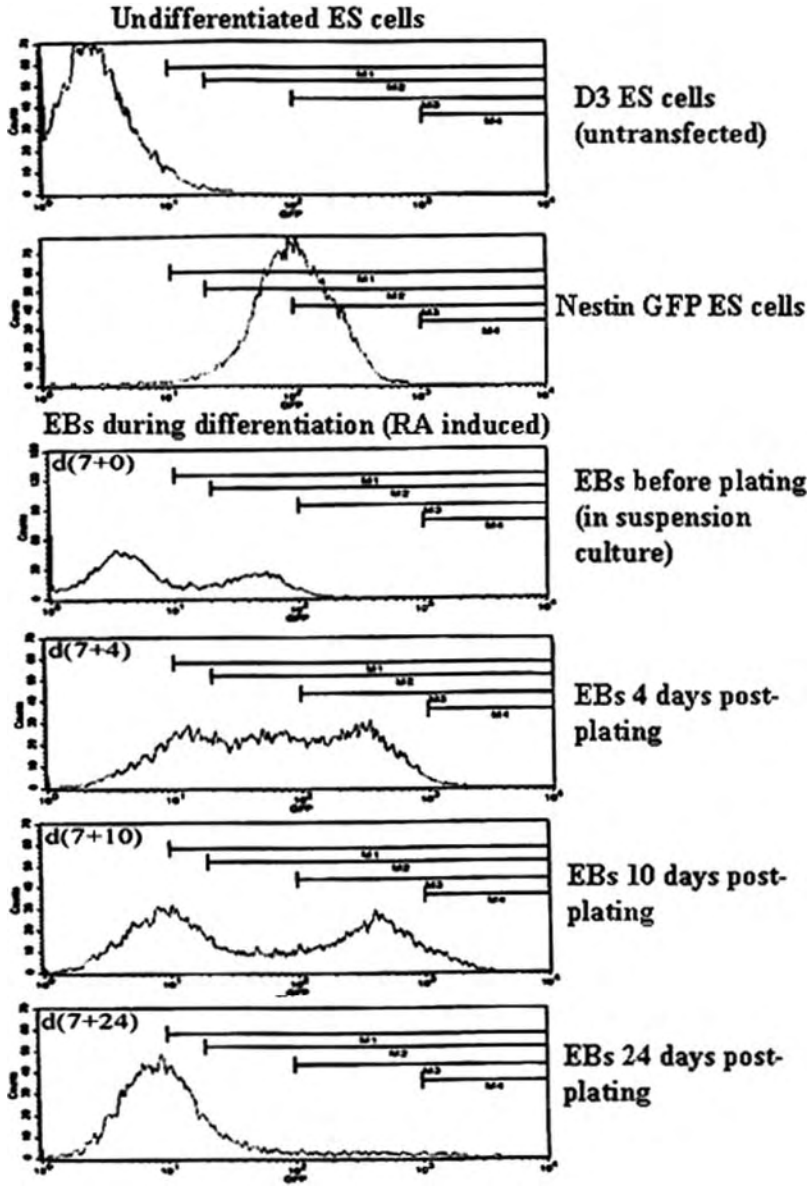

Fig. 4. Flow cytometry quantification of neural progenitors and differentiating neurons from nestin green fluorescent protein (GFP) embryonic stem cell clones. The *x*-axis represents the enhanced GFP (EGFP) intensity in logarithmic scale; the *y*-axis shows the number of cells. A comparative analysis of EGFP expression profile at various stages of differentiation reveals the generation of the maximum number of neural progenitors (the brightest shining ones) during the time window of 4–10 d of embryoid body plating. Subsequently there is a concurrent increase in the number of $EGFP^{-}$ cells deciphering the downregulation of endogenous nestin expression and

13. SmaI (NEB, cat. no. RO141L), HindIII (NEB, cat. no. RO104S), and NotI (NEB, cat. no. RO189S).
14. Ethidium bromide.
15. 1-kb and 100-bp DNA ladder.
16. Plasmid Medi kit (Qiagen, Hilden, Germany; cat. no. 12143).
17. DNA gel extraction kit (Qiagen; cat. no. 20021).
18. Polymerase chain reaction purification kit (Qiagen, cat. no. 28104).

2.4. Tissue Culture

1. Tissue culture dishes (35, 60, and 100 mm; 24, 48-well flat bottom and 96-well round bottom).
2. Graduated centrifuge tubes (15 and 50 mL).
3. Cryovials (2 mL).
4. Disposable pipets (5, 10, and 25 mL).
5. 100-mm bacteriological-grade dishes.
6. High-glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA; cat. no. 11960-069).
7. DMEM/F12 (1:1) (Invitrogen, cat. no. 21041-025).
8. Neurobasal medium (Invitrogen, cat. no. 21103-049).
9. ES cell-qualified fetal bovine serum (FBS; Invitrogen, cat. no. 10439-024).
10. L-Glutamine (200 m*M*; Invitrogen, cat. no. 25030-081).
11. Nonessential amino acid (100X; Invitrogen, cat. no. 11140-050).
12. Penicillin-streptomycin (pen-strep; 10,000 U/mL penicillin and 10 mg/mL streptomycin) (Invitrogen, cat. no. 15140-122).
13. ITS (insulin-transferrin-selenium) supplement (Invitrogen, cat. no. 51300-044).
14. B27 supplement (Invitrogen, cat. no. 17504).
15. Fibronectin (Invitrogen, cat. no. 12173-019).
16. Trypsin-1 m*M* EDTA (Invitrogen, cat. no. 25200-056).
17. Calcium- and magnesium-free phosphate-buffered saline (PBS) (pH 7.4; Invitrogen, cat. no. 10010-023).
18. PBS with calcium and magnesium (Invitrogen, cat. no. 14040-141).
19. Geneticin (G418; Amersham, Cleveland, OH; cat. no. 11379).
20. Mitomycin C (Sigma, cat. no. M-4287).
21. 2-Mercaptoethanol (Sigma, cat. no. M-7522).
22. Dimethyl sulfoxide (DMSO; Sigma, cat. no. D-2650).
23. Poly-L-ornithine (Sigma, cat. no. P-4957).
24. All-*trans* retinoic acid (RA; Sigma, cat. no. R-2625). Prepare (10^{-2} *M*) stock of RA in absolute ethanol and store in aliquots at –20°C either in amber tubes or in tubes wrapped with aluminum foil to prevent exposure to light (*see* **Note 1**). For working solution, dilute to 10^{-4} *M* (100-fold) with the ES cell medium as the operational concentration and store at –80°C in aliquots (avoid repeated freezing-thawing).
25. Gelatin (Sigma, cat. no. G-9391).
26. Laminin (Sigma, cat. no. L-6274).

Fig. 4. *(Continued)* decline in neural progenitor generation as reflected by EGFP expression profile. This also implies the increase in the number of differentiated neural progenies during the course of differentiation in addition to the other nonneural population. (Reproduced with permission from **ref. *3***.)

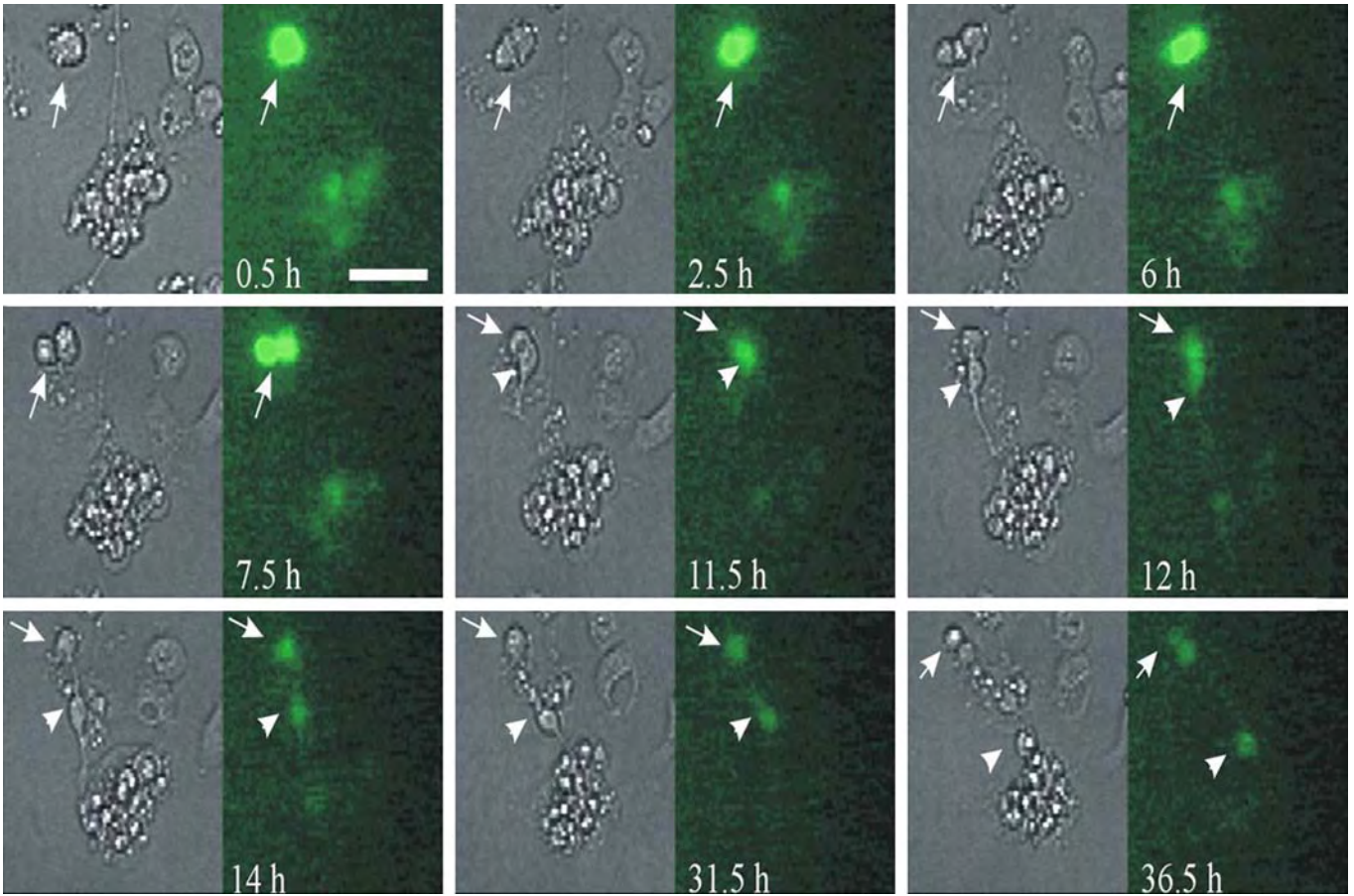

Fig. 5.

27. ESGRO (leukemia inhibitory factor, LIF; Chemicon International, Temecula, CA; cat. no. ESG 1107).
28. Collagenase B (Roche Applied Science, Mannheim, Germany; cat. no. 1088823). Prepare 0.1% collagenase solution in PBS, filter-sterilize, and store at –20°C in aliquots.
29. Cell dissociation buffer: for 100 mL, dissolve 634 mg KCl (85 m*M*), 523 mg K_2HPO_4 (30 m*M*), 123 mg $MgSO_4 \cdot 7H_2O$ (5 m*M*), 37 mg EDTA (1 m*M*), 276 mg Na_2ATP (5 m*M*), 55 mg Na-pyruvate (5 m*M*), 66 mg creatine (5 m*M*), 250 mg taurine (20 m*M*), and 360 mg glucose (20 m*M*) in water. Adjust to pH 7.2, filter-sterilize, and keep in aliquots at –20°C.

2.4.1. Medium

1. Fibroblast medium: DMEM supplemented with 10% FBS, 2 m*M* L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. Store at 4°C.
2. ES culture medium: DMEM supplemented with 2 m*M* L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 1X nonessential amino acids, 0.1 m*M* 2-mercaptoethanol, and 15% FBS. Store at 4°C. Add 1000 U/mL LIF directly to the dish after seeding the cells.
3. Differentiation medium: the same as in ES culture medium with 10% FBS and without LIF.
4. Defined medium for differentiation: this is a serum-free constituent that includes DMEM/F12 supplemented with 1X ITS supplement, 5 µg/mL fibronectin, 100 U/mL penicillin, and 100 µg/mL streptomycin. Store at 4°C.
5. Freezing mix: for 100 mL (2X) solution, mix 80 mL FBS with 20 mL DMSO. Store the solution at –20°C in aliquots. For immediate use, keep at 4°C and avoid repeated freeze-thawing.

2.5. Immunocytochemistry

1. Antinestin (Developmental Studies Hybridoma Bank, Iowa City, IA; cat. no. Rat 401).
2. Anti-SSEA-1 (Developmental Studies Hybridoma Bank, cat. no. MC480).
3. Anti-MAP2 (Sigma, cat. no. M4403).
4. Anti-tyrosine hydroxylase (Sigma, cat. no. T2928).
5. Anti-glial fibrillary acidic protein (anti-GFAP) (Sigma, cat. no. G3893).
6. Antisynaptophysin (Sigma, cat. no. S5768).
7. Anti-CNPase (Sigma, cat. no. C5922).
8. Anti-O4 (Chemicon, cat. no. MAB 345).
9. Goat antimouse Cy3 (Chemicon, cat. no. AP130C).
10. Goat antimouse Cy5 (Chemicon, cat. no. AP130S).
11. Goat serum (Sigma, cat. no. G6767).
12. Bovine serum albumin.
13. Tris-buffered saline (TBS): for 100 mL, dissolve 300 mg Tris-base, 20 mg KCl, and 80 mg NaCl in water, adjust to pH 7.4, and autoclave.

Fig. 5. The time-lapse monitoring of neural stem cell differentiation into neurons in nestin green fluorescent protein (GFP) clones. The live monitoring during differentiation reveals an asymmetric division (2.5, 6, and 36.5 h) of the neural stem cell/progenitor into a cell retaining the stem cell potential (arrow); the other one, because it is more specified, differentiates into a neuron (arrowhead) with a unipolar (11.5-h) and subsequent bipolar (12-h) morphology accompanied with cell migration and interaction with other cells in the vicinity (14 and 31.5 h). (Reproduced with permission from **ref. *3***.) (Please *see* the companion CD for the color versions of this figure.)

14. Paraformaldehyde (PFA): for a 4% solution, dissolve 4 g PFA (Sigma, cat. no. P6148) in 100 mL PBS with constant stirring using a magnetic stirrer and operating inside a fume hood (*see* **Note 2**). PFA goes into solution at 50–60°C. Cool it on ice, store at 4°C, and use within 1 wk.
15. Permeabilization solution: for 20 mL, dissolve 0.59 g ammonium chloride in PBS or TBS and add 50 µL Triton X-100 (Sigma). Either prepare fresh every time or store at 4°C to use within 1 wk.
16. Xylene.
17. Entellan (ProSciTech, Thuringowa Central, Australia; cat. no. IM022).
18. Cover slips (18 mm × 18 mm).
19. Glass slides.

2.6. Equipment and Accessories

1. Fluorescent inverted microscope (Nikon, Tokyo, Japan; model no. TE2000U).
2. Inverted microscope (Nikon, model no. TS100).
3. Confocal microscope (Carl-Zeiss, Oberkochen, Germany; model no. LSM510).
4. Biosafety cabinet (Thermo-Forma, model no. 1285).
5. Water-jacketed CO_2 incubator (Thermo-Forma, model no. 3121).
6. Hemocytometer/Neubauer chamber.
7. Liquid nitrogen container.
8. Gene Pulser II Electroporator System (Bio-Rad, Hercules, CA; model no. 165-2106).
9. 0.4-cm Genepulser cuvet (Bio-Rad, cat. no. 165-2088).
10. Flow cytometer (BD Biosciences, Franklin Lakes, NJ; model FACSCalibur).
11. Automated DNA sequencer (Applied Biosystems, Foster City, CA; model no. 3730).
12. Ultraviolet transluminator.
13. Agarose gel electrophoresis unit.
14. Ultraviolet spectrophotometer.
15. Water bath.
16. Orbital shaking incubator.

3. Methods

3.1. Nestin-GFP DNA (3)

1. Linearize the vector pEGFP1 (4.2 kb) using SmaI restriction digestion and gel purify using the Qiagen gel extraction kit according to the manufacturer's instructions.
2. Excise the region spanning human nestin intron II-tk (2 kb) basal promoter from the parent construct Nes 1852 tk/lacZ *(6)* using HindIII and NotI restriction enzymes.
3. Fill the staggered ends using dNTP mix and Klenow polymerase. Purify the 2-kb nestin product from the agarose gel using the Qiagen gel extraction kit according to the manufacturer's instructions.
4. Determine the concentration of both the vector (pEGFP1) and the insert (nestin) on an agarose gel by running side by side the known concentration of lambda DNA.
5. Set up individual reactions for blunt-end ligation using ligase buffer (1X), T4 DNA ligase, and varying molar ratios of vector and insert; incubate at 16°C overnight.
6. The next day, perform transformation using DH5/XL1-blue-competent bacteria using conventional heat shock approach *(19)* and grow on the LB-agar plate in the presence of 30 µg/mL kanamycin for 12–14 h in the orbital shaking incubator at 37°C.
7. Pick the resistant colonies individually and grow in separate tubes containing 2 mL LB liquid medium in the presence of kanamycin by incubating at 37°C overnight in the orbital shaking incubator with continuous shaking at 200 rpm.

8. Isolate the plasmid DNA from individual colonies using the widely used method of alkaline lysis *(19)* and subject the DNA to restriction digestion for screening for positive clones designated as h-nestin-EGFP (**Fig. 1**).
9. On verification of positive clones by restriction analysis on an agarose gel, reconfirm the insertion and orientation of the vector construct by dideoxy sequencing *(20)*.

3.2. Establishment of Nestin GFP Stable ES Cell Lines

3.2.1. Feeder Preparation

The mouse D3 ES cell line is cultured on mitotically inactive fibroblast feeders derived either from MEFs or STO cell line.

To prepare MEFs:

1. Sacrifice a pregnant mouse (12.5–14.5 dpc) by cervical dislocation and remove the placenta and fetal membranes under aseptic conditions.
2. Collect the embryos in a bacteriological dish containing PBS. Wash thoroughly with PBS and decapitate individual embryos. Dissect out the tail region, heart, and limbs, keeping the trunk region intact, which is subsequently minced into small pieces.
3. Collect the minced tissues in a sterile Erlenmeyer flask containing 5 mL trypsin-EDTA and stir continuously for 30–45 min at room temperature using a magnetic stirrer.
4. Filter the cell suspension through a sieve or sterilized cheesecloth and inactivate the trypsin with 10 mL fibroblast medium.
5. Pellet down the cells by centrifuging at 350*g* for 5 min and resuspend in fresh fibroblast medium. Seed about $1.5–2 \times 10^6$ cells/100-mm dish and incubate at 37°C and 5% CO_2 until they become 80–90% confluent (usually 2–3 d), and the passage is considered as zero (P0).
6. For subsequent propagation, dislodge the cells from the dishes by trypsinization, plate on the tissue culture dishes/flasks, and number the passages accordingly (*see* **Note 3**). The cells are also simultaneously frozen in freezing mix and maintained in the liquid nitrogen for future use (*see* **Subheading 3.3.**).

3.2.2. Mitotic Inactivation of Feeders

1. MEF or STO feeder cells, after attaining confluence, are either treated with mitomycin C or exposed to γ irradiation (12,000 rad) for mitotic inactivation.
2. For mitomycin C treatment, aspirate the medium from the dish and replenish with fresh medium sufficient to cover the cells (4–5 mL/100-mm dish).
3. Subsequently, add mitomycin C (10 µg/mL) to the dish, mix properly, and incubate in the CO_2 incubator at 37°C for 2.5 h (*see* **Note 4**).
4. Wash the cells thoroughly with PBS, trypsinize, plate onto the dishes (1×10^6 cells/60-mm dish), and use ideally within 1 wk of plating with intermittent changing of the medium.

3.2.3. ES Cell Thawing and Maintenance

1. Place a beaker with water at 37°C in a water bath.
2. Remove a vial of D3 ES cells stored in liquid nitrogen, loosen the cap inside a biosafety cabinet to release the pressure, and retighten the cap immediately.
3. Hold the vial upright inside the beaker until the contents show signs of melting (*see* **Note 5**).
4. Transfer the contents immediately under aseptic conditions inside a biosafety cabinet to a 15-mL sterile screw-cap tube containing 5–6 vol of ES culture medium to dilute the DMSO in the freezing mix.

5. Spin down the tube at 350*g* for 5 min, discard the supernatant, and suspend the cell pellet in 1 mL ES culture medium.
6. Seed the cells onto a mitotically inactivated fibroblast feeder monolayer in a 60-mm dish containing ES culture medium supplemented with LIF.
7. Incubate the dish at 37°C in an incubator with 5% CO_2.

3.2.4. ES Cell Maintenance

Murine ES cells are usually maintained in an undifferentiated state by culturing on fibroblast feeders that secrete the cytokine LIF in a paracrine fashion *(21)*. Recent reports indicated that the fibroblast also secretes BMP4 *(22)*, which has been proven to play a key role in ES cell self-renewal and maintenance of pluripotency *(23)*. Hence, the cells can also be maintained on gelatin-coated dishes in ES culture medium supplemented with LIF or LIF^+ BMP4 *(23)*. We usually maintain the cells on feeders in ES culture medium supplemented with 1000 U/mL LIF *(3)*. However, prior to differentiation we prefer to maintain the cells without the feeders on gelatin-coated dishes for a minimum of three passages.

1. Grow the ES cells until confluence.
2. Passage the cells ideally every 48 h using trypsinization and plating. The plating density, however, influences the subsequent maintenance of undifferentiated status and pluripotency of ES cells (*see* **Note 6**).

3.2.5. Transfection and Stable Clone Pick Up

For generation of stable clones, it is mandatory to linearize the vector DNA with intact promoter-reporter gene cassette, which needs to be incorporated and integrated stably into the genome. Ideally, we prefer using the unique site located upstream of the gene in the multiple cloning site of the vector backbone or the site located in the 5′ end of the gene used for cloning into the vector. The protocol used for generating nestin GFP-stable D3 ES cell lines follows.

3.2.5.1. Linearization of DNA Construct

1. Digest about 30 µg h-nestin-EGFP vector with HindIII restriction enzyme, which will linearize the plasmid required for stable integration of DNA following transfection.
2. Confirm the digestion on an agarose gel. Inactivate the enzyme at 65°C for 15 min and precipitate with absolute ethanol. At this point, the DNA will be easily visible as a string to the naked eye.
3. Spin down the DNA at 12,000*g* for 15 min at room temperature and wash the DNA pellet with 70% ethanol. Carry out the subsequent steps inside the biosafety cabinet.

3.2.5.2. Electroporation

For generating stable ES cell lines for nestin GFP, we followed the electroporation technique and selected the clones with G418.

1. Prepare a 100-mm dish with a monolayer of mitotically inactive neomycin resistant (neo^R MEF or STONeo) feeders, preferably on the previous day.
2. Aseptically, aspirate the 70% ethanol described in **Subheading 3.2.5.1.** and leave the tube open for 2–3 min to dry the DNA pellet (*see* **Note 7**).

3. Dissolve the linearized DNA in 50 µL sterile PBS and set aside.
4. Trypsinize a confluent dish of D3 ES cells and count the number of cells. In a single transfection experiment, use approx 3–5 × 10^6 cells for stable integration of h-nestin-EGFP DNA into the D3 ES cell genome.
5. Wash the cells twice with sterile PBS and suspend the cell pellet in 800 µL PBS.
6. Add the cells to the tube containing the DNA and mix properly by gentle trituration.
7. Collect the DNA-cell mixture in the electroporation cuvet without introducing any air bubbles and incubate it on ice for 5 min.
8. Place the cuvet in the electroporation chamber slot and set the electroporator at 240 V and 500 µF. Give an electric pulse to the cells by pressing the automatic charge and pulse buttons simultaneously and release the buttons when the beep sounds, signifying the completion of electroporation.
9. Remove the cuvet from the slot and chill it on ice for 15–20 min.
10. Suspend the cells properly by gentle trituration, transfer the contents to the dish containing neomycin-resistant feeders, and grow them in ES cell medium with 15% FBS and 1000 U/mL LIF.

3.2.5.3. Picking Up the Stable Nestin GFP Clones

1. Add G418 (300 µg/mL) to the electroporated ES cells after 48 h.
2. Monitor the cells every day for cell death and accordingly replenish with fresh medium supplemented with LIF and G418. After 10–12 d of G418 selection, the colonies should look distinct and visible even to the naked eye (*see* **Note 8**).
3. Mark the resistant colonies at the bottom of the dish, remove the medium, and add PBS.
4. Place the dish under the microscope inside the biosafety cabinet, pick the marked clones individually using a 10-µL pipet, and transfer to a 96-multiwell dish (1 colony/well) already containing 50 µL trypsin-EDTA (*see* **Note 9**).
5. Perform trypsinization in batches of four to eight colonies at a time depending on the speed and skill of picking (*see* **Note 10**).
6. Triturate the cells gently in trypsin for single-cell preparation and subsequently inactivate them by adding 150 µL ES cell medium with 15% FBS. Transfer the cells (200 µL volume) individually from each well to a 48-multiwell dish containing a monolayer of mitotically inactive neomycin-resistant feeders.
7. Incubate the cells at 37°C in an incubator with 5% CO_2 until they become confluent (usually 2–3 d maximum). Propagate the confluent clones further in a 24-multiwell dish on neomycin-resistant feeders (*see* **Note 11**).
8. Examine each well and mark the confluent ones for splitting/freezing.
9. Transfer some (three to five) of the confluent nestin GFP clones into 35- or 60-mm dishes (with feeders) for further propagation, freezing, and subsequent characterization. Freeze the remaining confluent clones from a 24-well dish at –80°C for future analysis. Because each clone grows at a different rate, each is considered a separate cell line and handled accordingly.
10. Maintain the G418 exposure throughout the propagation period of the nestin GFP ES cell clones.

3.3. Freezing of Nestin GFP Clones

1. Trypsinize the cells, suspend the cell pellet in ES culture medium with 15% FBS, and count the number of cells.
2. Take approx 1.5–2 × 10^6 cells/clone in a freezing vial, label properly stating the clone identity and the passage number, and chill it on ice. Add an equal volume of freezing mix to each vial and mix properly by pipetting (*see* **Note 12**).

3. Transfer the vials to a freezing box and keep at –80°C or to a controlled-rate freezer for slow freezing. The next day, transfer the vials to the liquid nitrogen storage container for long-term storage.

3.4. ES Cell Aggregation

Cell aggregation is the first step for the ES cell differentiation into various lineages during which the cells receive the required signals because of the cell–cell contact and the communication. This is accomplished by culturing the cells either directly in suspension *(1,24)* or in hanging drop followed by suspension culture *(3,18,25)*. The advantage of the hanging drop method lies in using a defined number of cells to initiate cell aggregation. This number can be controlled or varied according to the experimental outcome, and the influence of the number of cells on differentiation efficiency can be monitored easily compared to that with the cells cultured directly in suspension. Following aggregation, the ES cells form a three-dimensional structure designated as the embryoid body (EB).

3.4.1. Hanging Drop Preparation

1. Disperse the undifferentiated ES cells into a single-cell suspension by trypsinization.
2. Count the number of cells and keep aside the cells in the desired volume with a density of 500 cells/20 µL in differentiation medium.
3. Add 1 mL sterile PBS to a 100-mm bacteriological dish to help maintain the humidity and prevent drying of the hanging drops.
4. Keep the lid of the dish face up. Mix the cells in **step 2** properly and dispense the cells in a 20-µL volume to the inside of the lid. Normally, 75–80 drops can be made in a 100-mm dish (*see* **Note 13**).
5. Carefully place the lid with hanging drops back on the bottom dish with PBS. Incubate the dish at 37°C with 5% CO_2 for 2 d (*see* **Note 14**).
6. Collect the EBs in the hanging drop, grow in suspension culture in a 100-mm dish containing 8–10 mL of the same differentiation medium, and allow them to grow for 3–5 d in suspension to attain a reasonable size for plating.

3.4.2. Suspension Culture

1. Disperse the cells by trypsinization.
2. Seed about 1×10^6 cells into a 100-mm bacteriological dish containing 8–10 mL differentiation medium.
3. Incubate the cells at 37°C and 5% CO_2 for 3–5 d, during which the cells come together and form EBs of varying sizes depending on the number of cells/aggregate.

3.5. Neural Differentiation of Nestin GFP ES Cell Clones

We have attempted the neuronal differentiation from nestin GFP clones following two different protocols: by induction with RA *(3,26)* and by use of serum-free defined medium *(1,18)*. Both the protocols showed similar differentiation profiles in terms of the time window for optimum neural progenitor generation, determined quantitatively by flow cytometry. We prefer to take four to six clones initially for initiating differentiation to rule out the possibility of clonal variations in terms of EGFP expression and differentiation pattern. Subsequently, more clones are thawed

and analyzed. Eventually, one of the representative clones is selected for further investigation.

3.5.1. Induction With RA

RA is the vitamin A derivative with a pleiotropic effect during early embryonic development; it manifests its influence through its receptors RAR and RXR *(27)*. For neural differentiation, RA seems to have a profound effect, supposedly in a concentration-dependent manner, in suppressing mesodermal genes and inducing ectoderm from which the neuroectoderm arises *(28)*. Add RA directly either to the cells suspended in the medium for preparing hanging drops or to the EBs in suspension culture to make the final concentration of RA 100 n*M* to 1 µ*M*. We have also tried adding RA during plating of the EBs for 2 d consecutively, and irrespective of the time of RA exposure, the results remained comparable with respect to the nestin-specific EGFP expression and neural differentiation.

3.5.2. Plating of the EBs and Monitoring Neural Differentiation

1. Add the differentiation medium to each gelatinized or polyornithine-laminin-coated dish (800 µL to 1 mL/24-multiwell dish; 1.5 mL/35-mm or 3–5 mL/60-mm dish).
2. Pick the EBs on d 3–5, which correspond to differentiation d 5–7 (2 d in hanging drop and next in suspension), that are of a reasonable size and are visible to the naked eye as globular particles suspended in the medium and then plate 5–10 EBs/35-mm dish.
3. Monitor the plated EBs daily under the inverted microscope and record the events using both bright field and fluorescence. The EBs show a centrifugal growth pattern, and the appearance of cells with epithelial morphology in the EB outgrowth signifies the neural progenitor population that are EGFP positive as well.
4. Change the medium every 48 h.
5. Fix the cells at different days postplating and perform immunocytochemistry (*see* **Subheading 3.6.**) to authenticate the neural-specific expression of EGFP under the regulatory control of nestin intron II in the nestin GFP clones.
6. In addition to the qualitative monitoring of neural differentiation and EGFP expression profile, the flow cytometric quantification (*see* **Subheading 3.7.**) substantiates the number of neural progenitors generated following the said protocol and deciphers the optimum time window for their generation.

3.5.3. Neural Differentiation Using Defined Medium

1. Plate EBs (5–7 d old) on gelatin/polyornithine-laminin-coated dishes using differentiation medium for the EBs to adhere to the dish.
2. The next day, change the medium to defined medium and culture for 6–7 d. At this time, the $EGFP^+$ cells with epithelial morphology are distinctly visible.
3. Dissociate the cells by trypsinization and either plate the cells in defined medium supplemented with 10 ng/mL basic FGF for enrichment of $EGFP^+$ neural progenitors or grow them in N2 basal medium with B27 supplement for differentiation into neural cells. The basic FGF-exposed cells for 4–6 d can be grown further in N2 medium for differentiation.
4. Monitor the cells daily for differentiation into neurons, astrocytes, and oligodendrocytes. As described in **Subheading 1.**, during the course of differentiation the $EGFP^+$ neural progenitors will give rise to more mature neurons that are $EGFP^-$, signifying the downregulation of nestin in these cell populations.

3.6. Immunocytochemistry

The specificity of nestin intron II-driven EGFP expression in the nestin GFP clones as well as the identity of differentiated neural derivatives were verified by immunocytochemistry using various antibodies against neurons and glia.

1. Grow the cells on glass cover slips.
2. Remove the medium, wash the cells with PBS, and fix using 4% PFA for 20 min at room temperature.
3. Wash the cells twice with PBS and permeabilize for 10 min (*see* **Note 15**).
4. Wash the cells with PBS or TBS three times for 5–10 min each.
5. Prevent the nonspecific binding of the antibodies by preincubating the cells with 5% goat serum and 0.8% bovine serum albumin for 1 h (*see* **Note 16**).
6. Incubate the cells with the respective primary antibodies (antinestin for detecting neural stem cells/progenitors, anti-MAP2 for mature neurons, antisynaptophysin for neurons showing synaptic connections, anti-GFAP for astrocytes, anti-O4 or anti-CNPase for oligodendrocytes, anti-tyrosine hydroxylase for dopaminergic neurons, anti-5HT for serotonergic neurons) for 2–3 h at room temperature with gentle agitation (*see* **Note 17**).
7. Wash the cells thoroughly with TBS three times for 10 min each.
8. Incubate the cells with fluorochrome (Cy3/Cy5)-conjugated secondary antibody for 1 h at 37°C for fluorescent labeling (*see* **Note 18**).
9. Repeat **step 7** to remove all the unbound fluorochromes from the cells.
10. Dehydrate the cells by exposing to ethanol gradients (70, 90, 100%) in quick succession (3–4 s each) followed by xylene exposure for 3–4 s.
11. Mount the cover slips on glass slides using entellan, allow to dry, and store in the dark at 4°C until observation.
12. Observe the slides under a fluorescent or a confocal microscope to detect EGFP as well as Cy3/Cy5 labeling.

3.7. Flow Cytometric Quantification

The EGFP expression profile of nestin GFP clones envisaging the neural development and differentiation pattern from ES cells is quantitatively ascertained by flow cytometry using an FACSCalibur flow cytometer equipped with a 488-nm argon ion laser (15 mW) *(3,18)*. Because the committed neural cells from nestin GFP clones during differentiation exhibit varying levels of EGFP in concordance with endogenous nestin expression, the cells can be categorized as neural progenitors or differentiating neural derivatives and quantified based on the number of cells residing within a specific intensity range. Although the cells exhibiting the brightest EGFP expression signify the neural stem cells/progenitors, the weak positive ones can be considered as differentiating early neurons and glial cells. The more mature neural cells as well as the nonneural ones will be EGFP negative. This pattern is very well reflected in the flow cytometric quantification (*3*; **Fig. 4**).

1. Trypsinize the nestin GFP clones at various developmental stages (undifferentiated ES cells, EBs in hanging drop, suspension culture, and different days postplating) by incubating at 37°C for 2–5 min.
2. Gently triturate to make a single-cell suspension and inactivate trypsin by adding differentiating medium. Collect the cells in separate tubes.

3. In a similar manner, process the wild-type (untransfected) ES cells for setting the data acquisition parameters in the flow cytometer as the negative control.
4. Spin down the cells at 350*g* for 5 min and wash twice with PBS.
5. Resuspend the cells by gentle trituration using 500 µL PBS containing Ca^{2+} and Mg^{2+} (*see* **Note 19**).
6. Switch on the flow cytometer and run the negative control sample for parameter settings to detect the scattering pattern (forward and side scatter) to keep the control peak below 10^1 log scale by adjusting the voltage and ampere settings and the threshold values.
7. Acquire the samples from both control and nestin GFP cells without changing any of the settings. Measure the emitted fluorescence of EGFP in log scale at 530 nm (fluorescein isothiocyanate bandpass filter) and perform the analysis using *CellQuest* software.

3.8. Single-Cell Preparation for Time-Lapse Monitoring and Patch-Clamp Recordings

1. Mechanically dislodge the plated EBs at various time-points using either a sterile blunt-end needle or a 10-µL pipet tip.
2. Collect the cells in a 15-mL screw-cap tube containing PBS.
3. Allow the cells to either settle down or spin them down at 350*g* for 5 min. Aspirate the PBS and add 200 µL collagenase B.
4. Incubate the cells for 30 min in the CO_2 incubator at 37°C, keeping the cap loosened slightly.
5. Spin down the cells at 350*g* for 5 min and replace the collagenase with 200 µL cell dissociation solution.
6. Stir the cells slowly for 30 min at room temperature using a magnetic stirrer, add 200 µL differentiation medium, and suspend by gentle trituration.
7. Add the differentiation medium to dishes with gelatin-coated glass cover slips.
8. Plate the cells onto the cover slips carefully in a dropwise manner. Plating volume can be determined empirically depending on the desired cell density. Incubate the cells overnight at 37°C in a CO_2 incubator.
9. The next day, monitor the cells under the microscope for attachment to the cover slip and growth. Replenish with fresh medium. These single isolated cells can be used further for electrophysiological investigations (patch-clamp recordings; ***3***) and immunocytochemical characterizations within 2–5 d of plating and for real-time monitoring of neural differentiation after 12–24 h plating using time-lapse video microscopy ***(3)***.

4. Notes

1. Because RA is light sensitive, direct exposure to light is avoided.
2. Care should be taken not to inhale the fumes; either a bottle with a lid or a conical flask with the mouth covered with aluminum foil should be used.
3. Unlike STO cells, the MEFs used as feeders are most effective when used up to passage number P6/P7 maximum. When using STO cells as feeders, the propagation is done in the same manner as described for MEFs.
4. Mitomycin C is light sensitive and highly toxic. It should be handled in the dark, and gloves should be worn while handling it.
5. Care should be taken not to thaw it completely as the DMSO will be toxic to the cells. The principle of slow freezing and rapid thawing is ideal for postfreezing cell viability.
6. The seeding density during propagation depends on the growth rate of the ES cells because too much or too little growth leads to differentiation of cells, resulting in the loss of pluripotency.

Ideally, the cell density during plating should be reflective of the density that is attained after 48 h postplating when the cells show 80–90% confluency. We usually use approx 0.1–0.2 × 10^6 or 0.25–0.4 × 10^6 cells, respectively, on a 35-mm dish with gelatin coating or with feeders.

7. Care should be taken not to overdry the DNA; otherwise, it would be difficult to dissolve.
8. Starting at d 2 of exposure of cells to G418, many cells will be floating detached from the plate, indicating the G418 sensitivity of the cells that have not taken up the transfected h-nestin-EGFP vector DNA having the neomycin cassette. The majority of cell death is observed by 1 wk of exposure, and it is ideal to change the medium daily during this period with LIF and G418. During the remaining 3–5 d (i.e., d 10–12 of G418 selection), the medium can be changed every other day.
9. Keep a new box with 10-µL tips open to be used to pick and transfer individual colonies to corresponding wells of the 96-well dish (each row will exactly match the same in a 96-well dish). This would curtail the confusion in picking and transferring the clones for trypsinization. A similar strategy can also be followed while transferring the clones from a 96- to a 48-well dish after trypsinization by using the tips from a 200-µL box in which 8 wells of a 48-well dish correspond with the 8 wells of the box in a horizontal direction.
10. Exposure to trypsin for more than 3–4 min might affect the cell membrane integrity, which would result in cell death.
11. There might be asynchronous growth of cells during this time. Hence, only the clones that have attained confluency are propagated first, and the remaining ones are propagated on subsequent days, depending on their growth rate.
12. In case of freezing cells from a 24-well dish, those can be frozen in the dish itself or in the vial as described and can be kept at –80°C until further characterization is done.
13. Care should be taken not to have the drops close to the rim of the lid to prevent contamination or too close to each other, leading to "drop merger".
14. This helps the cells come together and form the EBs that remain floating under the influence of gravity.
15. For cell surface antigens, permeabilization is not required.
16. FBS can also be used for blocking the nonspecific binding in place of goat serum.
17. In each case, the negative control should be performed with the substitution of respective primary antibodies with either preimmune serum or serum immunoglobulin G.
18. Fluorochromes are light sensitive; hence, direct exposure to light should be avoided.
19. Care should be taken to not have clumps or to damage the cells by vigorous mixing because both will adversely affect the measurement. If necessary, use a sieve to get rid of clumps that would otherwise block the nozzle of the flow cytometer.

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4

Optimized Neuronal Differentiation of Murine Embryonic Stem Cells

Role of Cell Density

Matthew T. Lorincz

Summary

Neuronally differentiated embryonic stem (ES) cells offer a flexible and extremely potent model to study nervous system development and disease. A variety of protocols have been described to facilitate neuronal differentiation. The density of ES cells used for neuronal differentiation has striking effects on the proportion and purity of the derived neuronal cells. Here, the protocols used to optimize ES cell density in neuronal differentiation with and without an initial aggregation step are described.

Key Words: Cell density; embryoid bodies; embryonic stem cells; monolayer neuronal differentiation; neuronal differentiation.

1. Introduction

Neuronally differentiated embryonic stem (ES) cells offer a flexible and extremely potent model to study nervous system development and disease. Stem cells have additional therapeutic promise for neurodegenerative diseases. A large number of protocols have been devised to facilitate neuronal differentiation of ES cells with varying degrees of success. The resulting cultures are typically heterogeneous and at times difficult to reproduce.

Classically, formation of embryoid bodies (EBs) has been the starting point for most neuronal differentiation protocols. EBs are small aggregates of ES cells that form in suspension culture. In the frequently utilized $4^{-}/4^{+}$ protocol ***(1)***, EB formation is followed by EB plating and production of cultures that are 40–50% neuronal and contain endoderm and mesoderm derivatives ***(2)***. The neuronal cells produced by the $4^{-}/4^{+}$ protocol are glutamatergic (approx 70–80%), GABAergic (25%), and glycinergic (5%). The glial cells are primarily astrocytic ***(3,4)***. Treatment of EBs with conditioned medium from the hepatocarcinoma cell line (HepG2) increases the

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proportion of cellular aggregates expressing neuronal markers to 80% while dramatically reducing markers of endoderm and mesoderm *(5)*. Growth of ES cells on stromal cell lines such as PA6 also increases neuronal differentiation. Approximately 50% of ES cells co-cultured with PA6 cells express neuronal markers; of the neurons, 20–30% express markers of midbrain dopamine neurons, the cell type primarily lost in Parkinson's disease *(6)*.

Use of EBs in multistep protocols in which EBs are subjected to neuronal precursor selection, and subsequent expansion in the presence of fibroblast growth factor (FGF) 2, FGF-8, Sonic hedgehog (Shh), or ascorbic acid has been shown to enhance the efficiency of neuronal differentiation (50–70%) and in the case of FGF-8 and Shh promote midbrain dopamine neuron production *(7,8)*. Within EBs, stochastic cell-to-cell interactions (i.e., Notch/Delta and β-catenin signaling pathways) and soluble factors (i.e., leukemia inhibitory factor [LIF] and FGFs) produce unique environments that influence cell fate in an unpredictable manner and has led to the development of alternative neuronal differentiation protocols *(9,10)*. Genetic manipulation of ES cells to enhance neuronal differentiation has the advantage of producing a higher proportion of neurons and neuronal subtypes but does so at the expense of overriding endogenous differentiation pathways *(11,12)*. Alternative approaches that bypass formation of cellular aggregates or co-culture have been described to produce cultures 80–90% neuronal *(13,14)*.

Regardless of the protocol utilized to promote neuronal differentiation, cell density is a critical, although often ill-defined, variable. In the original description of the $4^{-}/4^{+}$ protocol, the cell number for production of EBs was defined as one-quarter of a confluent T-25 flask, and EBs were described as being plated at a density of "about 100" per 35-mm well. An exact cell number and medium volume may be given for part of a protocol, such as for the formation of EBs, but the density of EB plating was not described *(3)*. In other frequently cited protocols, there is no definition of cell number, or cell number is referenced to a text that may be difficult to obtain *(8,15)*. At times, a defined number of cells is described, but the volume of medium is not, so the critical variable of cell density is left undefined *(12,16)*. Some protocols describe relatively wide cell density ranges in which the low and high densities produce strikingly different results *(9,14)*.

The intent of this series of investigations was to test the role of cell density in neuronal differentiation with the goal of optimizing densities for production of neurons with and without an initial aggregation step. It is important to point out that the critical cell number variable for production of cellular aggregates is the number of cells per volume of medium. This is in contrast to the plating of cellular aggregates or differentiation of ES cells in monolayer, for which the key density variable is cell number per area of tissue culture plastic.

2. Materials

2.1. Tissue Culture

2.1.1. Routine ES Cell Culture and Storage

1. T-75 flasks with filter caps (Corning, Corning, NY; cat. no. 430641).
2. T-25 flasks with filter caps (Corning, cat. no. 430639).
3. 15-mL centrifuge tubes (BD Falcon, Franklin Lakes, NJ; cat. no. 352095).

4. 50-mL tubes (BD Falcon, cat. no. 352098).
5. Freezing vials (Corning, cat. no. 430659).
6. Sterile pipets (2, 5, and 10 mL; Fisher, Hampton, NH; cat. no. 357507, 13-678-11D/13-678-11E, and 13-678-11E, respectively).
7. Bottle-top filters (Nalgene, Rochester, NY; cat. no. 166-200).
8. Media bottles (100, 250, and 500 mL; Corning, cat. no. 431175, 430281, and 430282, respectively).
9. Nalgene cryo 1°C freezing container (Nalgene, cat. no. 5100-0001).
10. 1% gelatin solution: dissolve 5 g gelatin (Sigma, St. Louis, MO; cat. no. G-1890) in 500 mL sterile water (Sigma, cat. no. W-3500) followed by autoclaving. Dissolve 55 mL 1% gelatin in 500 mL sterile water to produce a 0.1% gelatin solution that is placed on tissue culture plastic at a volume adequate to completely cover the surface (T-25 = 3 mL; T-75 = 5 mL). Gelatin is left in contact with the tissue culture plastic for at least 20 min at 4°C prior to use, then removed and medium added.
11. ES supplement: add 23.83 g HEPES (Gibco, Carlsbad, CA; cat. no. 11344-025), 4 g L-glutamine (Gibco, cat. no. 21051-016), and 70 µL 2-mercaptoethanol (Sigma, cat. no. M-7522) to a total volume of 1 L Dulbecco's modified Eagle's medium (DMEM); 28-mL aliquots of ES cell supplement can be stored at –80°C.
12. ES growth medium: ES cells are maintained in supplemented DMEM medium. The DMEM (Gibco, cat. no. 11960-044) is 10% fetal bovine serum (ES tested; Atlanta Biologicals Premium Select, Lawrenceville, GA; cat. no. S11550). Add 28 mL ES cell supplement per 500 mL ES cell medium. LIF (Chemicon, Temecula, CA; cat. no. 11161030) is added at 1000 U/mL to fresh medium *(17)*.
13. Ca^{++}-/Mg^{++}-free 10X Hanks balanced salt solution (HBSS; Gibco, cat. no. 14180-061) is diluted to 1X in water.
14. 2.5% trypsin (Sigma, cat. no. 15400-054) is diluted to 0.25% in 1X Ca^{++}-/Mg^{++}-free HBSS. Add 200 µL 0.5 *M* ethylenediaminetetraacetic acid (Gibco, cat. no. 15575-038).
15. Freezing/storage medium: 90% fetal bovine serum/10% dimethyl sulfoxide (Sigma, cat. no. D-2650).

2.1.2. Neuronal Differentiation

2.1.2.1. Modified Neurosphere Differentiation

1. T-25 flasks with filter caps (Corning, cat. no. 430639).
2. 60- or 100-mm tissue culture dishes (BD Falcon, cat. no. 35-3004 and 35-3003, respectively) exposed to ultraviolet (UV) light for 24 h to produce a sterile, minimally adhesive surface.
3. 6- or 12-well tissue culture plates (Corning, cat. no. 3506 and 3512, respectively).
4. 80:20 neuronal differentiation medium: combine 80 mL F12 medium (Invitrogen, Carlsbad, CA; cat. no. 11765-054), 800 µL N2 salts (Invitrogen, cat. no. 15704), 20 mL neurobasal medium (Invitrogen, cat. no. 21103-049), 400 µL B27 salts (Invitrogen, cat. no. 15704), and 1 mL 100 m*M* pyruvate (Invitrogen, cat. no. 11360-070) (*see* **Note 1**).
5. N2:B27 neuronal differentiation medium: combine 50 mL neurobasal medium, 1 mL B27 supplement, 50 mL DMEM/F12 (Invitrogen, cat. no. 11330-032), 500 µL N2 supplement, 33.5 µL bovine serum albumin fraction V (Invitrogen, cat. no. 15260-037), and 1 mL 100 m*M* pyruvate (*see* **Note 1**). Retinoic acid in addition to that contained in the B27 medium can be added. The addition of 25.5 µL of a 2 m*M* retinoic acid stock provides an additional 0.5 µ*M* concentration (*see* **Notes 2** and **3**).

6. Polyornithine (Sigma, cat. no. P-8638): working solution: 0.01% polyornithine diluted in water.
7. Laminin (Gibco, cat. no. 23017-015): working solution is 20 ng/mL laminin dissolved in water.

2.1.2.2. Monolayer Neuronal Differentiation

1. T-25 flasks with filter caps (Corning, cat. no. 430639).
2. 6-, 12-, and 24-well tissue culture plates (Corning, cat. no. 3506, 3512, and 3524, respectively).
3. Thermanox cell culture cover slips (13 mm; Nunc, Rochester, NY; cat. no. 174950).
4. N2:B27 medium (*see* **Subheading 2.1.2.1., item 5**).
5. Polyornithine (*see* **Subheading 2.1.2.1., item 6**).
6. Laminin (*see* **Subheading 2.1.2.1., item 7**).

2.2. Analysis of Differentiation

2.2.1. Immunohistochemistry

1. 1X phosphate-buffered saline (PBS; Gibco, cat. no. 10010-023).
2. 3.7% formaldehyde (Mallinckrodt, Hazelwood, MO; cat. no.5016) in 1X PBS.
3. 0.5% Triton X-100 (Sigma, cat. no. T9284) in 1X PBS.
4. Primary antibodies:
   a. Murine monoclonal antibody against neuronal class III tubulin (Covance, Berkekey, CA; cat. no. 435P) diluted 1:250 in 1X PBS containing 10% serum.
   b. Goat polyclonal antibody against Oct4 (Santa Cruz Biotechnology, Santa Cruz, CA; cat. no. SC-8628) diluted 1:100 in 1X PBS containing 10% serum.
5. Secondary antibodies:
   a. Cy3 conjugated affiniPure donkey antimouse immunoglobulin G(H + L) (Jackson Immuno Research, West Grove, PA; cat. no. 715-165-151) diluted 1:150 in 1X PBS containing 10% donkey serum.
   b. Fluorescein isothiocyanate (FITC)-conjugated affiniPure donkey antigoat immunoglobulin G(H + L) (Jackson Immuno Research, cat. no. 705-095-147) diluted 1:100 in 1X PBS containing 10% donkey serum.
6. 5 µg/mL Hoescht 33258 (Sigma, cat. no. B-1155) in 1X PBS.
7. Glass slides (Clay Adams, Hampton, NH; cat. no. 3050).
8. Glass cover slips number 1 (22-mm^2; Corning, cat. no. 2865-22).
9. Mounting medium such as gel/mount (Biomedia, Foster City, CA; cat. no. MO1).

2.2.2. Reverse Transcription Polymerase Chain Reaction

1. Trizol (Invitrogen, cat. no. 15596-018).
2. Chloroform (Sigma, cat. no. C2432).
3. Ethanol (Pharmco, Brookfield, CT; cat. no. 64-17-5).
4. Agarose (Invitrogen, cat. no. 15510-019).
5. Superscript (Invitrogen, cat. no. 18064-071).
6. Oligo dT 13mers (Invitrogen, cat. no. 18418-012).
7. Deoxyribonuclease I (Invitrogen, cat. no. 18068-015).

2.3. General Equipment

1. Centrifuge (International Clinical Centrifuge [IEC], model no. CL).
2. Cell freezer (–140°C chest freezer or liquid nitrogen storage tank).

3. Humidified tissue culture CO_2 incubator.
4. Tissue culture hood.
5. Inverted microscope.
6. Coulter particle counter or hemocytometer.
7. Vacuum pump.
8. Rocking platform.
9. Thermocycler.
10. Spectrophotometer.

3. Methods

3.1. Routine ES Cell Culture and Storage

Excellent descriptions of routine ES cell culture and storage can be found elsewhere in this volume. The technique employed in these studies is described in detail in Embryonic Stem Cell Protocols, Volume 1, Chapter 16.

3.2. Modified Neurosphere Differentiation

3.2.1. Preparation of Polyornithine- and Laminin-Treated Dishes

1. Incubate tissue culture plastic with 0.01% polyornithine solution for 4–6 h at room temperature.
2. Remove polyornithine solution and sterilize plates by exposing for approx 18 h to UV light in a tissue culture hood.
3. Incubate dishes with 20 ng/mL laminin solution at 4°C for longer than 48 h. For a six-well plate, 1.5–2 mL polyornithine and laminin are used per well.
4. Completely remove laminin just prior to addition of cells (*see* **Note 4**).

3.2.2. Modified Neurosphere Differentiation

ES cells are initially plated onto a minimally adhesive surface that promotes formation of small cellular aggregates or "neurospheres," which are then plated onto an adhesive surface and allowed to differentiate ***(17)***. In this protocol, cell density is critical in both formation (cells/medium volume) and plating of the neurospheres (neurospheres/square centimeter surface area). Neurosphere formation has been carried out at cell concentrations ranging from 1×10^5 to 5×10^6 cells/mL (*see* **Table 1**). At densities greater than 8×10^5 cells/mL, large branching trabecular networks form instead of the desired neurospheres. Below a cell density of 3×10^5 cells/mL, small cell aggregates without a cavity form instead of the hollow-cavity neurospheres. A concentration of $4–6 \times 10^5$ cells/mL medium optimizes neurosphere formation and subsequent neuronal differentiation (*see* **Note 5**).

In addition to the importance of cell density (cells/milliliter) in formation of neurospheres, the density at which the neurospheres are plated (cells/square centimeter tissue culture plastic) is also important. The application for which neuronal differentiation is undertaken will ultimately determine optimal neurosphere plating densities. The following numbers can serve as a starting point, which can be optimized for individual applications. In general, below a certain neurosphere plating density few cells survive; at very high neurosphere plating densities, the cultures become overly dense, and a larger proportion of nonneuronal cells occupies the culture (*see* **Table 1**).

Table 1
Summary of Optimal Cell Density for Neuronal Differentiation

Neurosphere formation density (cells/mL)	$<3 \times 10^5$	$4–6 \times 10^5$	$>8 \times 10^5$
Result	Small cell aggregates	Optimal neurosphere formation	Large branching trabecular networks
Neurosphere plating density (neurosphere cells/well; *see* text for details)	$<2.5 \times 10^5$	$5–7.5 \times 10^5$	$>1.5 \times 10^6$
Result	Minimal survival	Optimal neurosphere plating density	Dense culture with increased nonneuronal differentiation
Monolayer ES cell differentiation without replate (cells/cm^2)	$<0.5 \times 10^4$	$0.8–1 \times 10^4$	$>1.5 \times 10^4$
Result	Minimal survival	Optimal ES cell plating density	Dense culture with increased nonneuronal differentiation
Replating cell density (d 4) (cells/cm^2)	$<2.5 \times 10^4$	$4–5 \times 10^4$	$>7.5 \times 10^4$
Result	Minimal survival	Optimal cell plating density	Dense culture with increased nonneuronal differentiation

Cultures in which neurospheres are formed at 5×10^5 cells/mL and then plated at a density of $5–7.5 \times 10^5$ will produce cultures in which greater than 90% of the resulting cellular aggregates undergo extensive neuronal differentiation. Cultures have been maintained in this manner for longer than 2 mo. Plating less than 2.5×10^5 neurosphere cells per well in a six-well plate generally results in almost total cell death. At densities greater than 1.5×10^6 neurosphere cells per well in a six-well plate, the resulting culture is extremely dense, individual cells are not readily identifiable, and the proportion of nonneuronal cells increases (*see* **Fig. 1A**,**B**).

1. Maintain ES cells in ES cell growth medium in T-25 or T-75 flasks to 70–90% confluency.
2. Routinely remove cells from their substrate with a 1X HBSS wash (T-25 = 3 mL; T-75 = 5 mL) and 0.25% trypsin (T-25 = 2 mL; T-75 = 4 mL) treatment for approx 1 min.
3. Place cells into a 15-mL conical tube containing a volume of ES growth medium (without LIF) equal to the volume of trypsin utilized. Centrifuge (1.5 min at a setting of 4 on the IEC centrifuge or equivalent; approx 1500*g*), remove supernatant and resuspend in a total volume of 2 mL 80:20 or N2:B27 medium.
4. Remove an aliquot of cells to IsotonII diluent in a Beckman Z1 Coulter particle counter and count cells (*see* **Note 6**).
5. To produce neurospheres, following counting evenly distribute cells in 2 mL medium by gently pipetting with a 2-mL pipet. Plate the desired volume of cells to obtain the appropriate

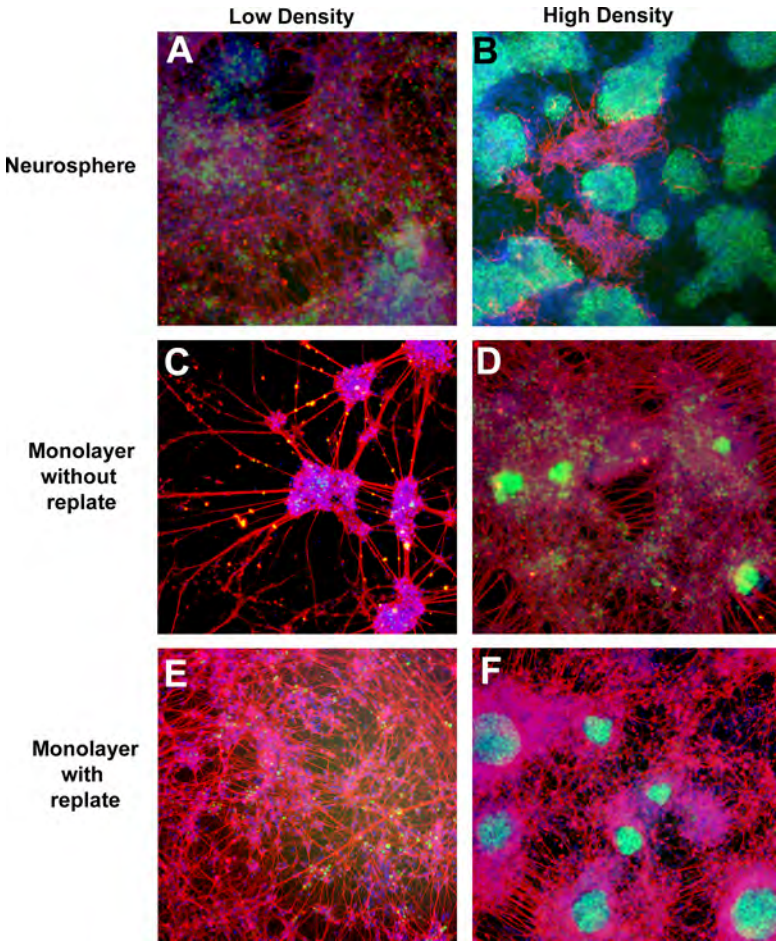

Fig. 1. Density-dependent neuronal differentiation of R1 embryonic stem (ES) cells. (**A**,**B**) Neurosphere neuronal differentiation. (**A**) Neurospheres were plated at a density of 7.5×10^5. (**B**) Neurospheres were plated at a density of 3.3×10^6. ES cells were neuronally differentiated utilizing the neurosphere differentiation protocol for 14 d and double labeled with βIII tubulin and Oct4. βIII tubulin expression is robust at both densities, as demonstrated by red CY3 conjugated secondary antibody staining. Oct4 expression increases dramatically at the higher neurosphere plating density, as demonstrated by green fluorescein isothiocyanate-conjugated secondary antibody signal. (**C**,**D**) Monolayer neuronal differentiation without replating. (**C**) ES cells were plated at a density of 0.8×10^4 cells/cm^2. (**D**) ES cells were plated at a density of 2.6×10^4 cells/cm^2. ES cells were neuronally differentiated for 14 d and double labeled with βIII tubulin and Oct4 as in **A** and **B**. Extensive neuronal differentiation is apparent at both plating densities. At the higher plating density, there is increased Oct4 staining. (**E** and **F**) Monolayer differentiation with replating. ES cells were initially plated at a density of 0.8×10^4 cells/cm^2. On d 4, cells were replated at a density of (**E**) 5×10^4 cells/cm^2 and (**F**) 15×10^4 cells/cm^2. ES cells were differentiated an additional 10 d and double labeled with βIII tubulin and Oct4 as above. Extensive neuronal differentiation is apparent at both plating densities. At the higher plating density, there is increased Oct4 staining. Blue Hoescht 33258 is also shown. **A–D**, ×10 magnification; **E** and **F**, ×5 magnification. (Please *see* the companion CD for the color versions of this figure).

cell number per milliliter onto 60- or 100-mm tissue culture dishes that were previously UV exposed overnight to produce a minimally adhesive substrate (*see* **Note 7**).
6. Incubate the cultures overnight at 37°C and 5% CO_2; the ES cell suspension forms small, uniform aggregates of cells, neurosphere-like clusters.
7. After 24 h, remove the medium containing the neurospheres to 15-mL conical tubes (*see* **Note 8**).
8. Gravity centrifuge the neurospheres for 20–30 min at room temperature.
9. Carefully remove the supernatant with a glass Pasteur pipet attached to a vacuum and gently resuspend the neurospheres in medium to avoid disassociation of the cellular aggregates.
10. To plate neurospheres; the desired volume of neurosphere suspension (*see* **Table 1**) is placed onto polyornithine- and laminin-treated tissue culture plastic in a total volume of 1.5–2 mL medium per well of a six-well plate (*see* **Note 9**). Neurosphere adherence is nearly complete after 24 h in culture.
11. Incubate cultures in 5% CO_2 at 37°C. Change the medium the following day and every other day thereafter with 2 mL medium. Cultures are routinely maintained for 14 d, following which a high proportion of neuronal cells are present (*see* **Fig. 1A**,**B**).

3.3. Monolayer Neuronal Differentiation

3.3.1. Preparation of Polyornithine- and Laminin-Treated Cover Slips

1. Prepare polyornithine- and laminin-treated six-well tissue culture plates as described (*see* **Subheading 3.2.1.**). However, 13-mm round, unwashed Thermanox cell culture cover slips can be added to 6-, 12-, or 24-well plates prior to polyornithine and laminin treatment.
2. Place up to four 13-mm round cell culture cover slips in a 6-well plate chamber or one 13-mm round cover slip in either a 12- or a 24-well chamber (*see* **Note 10**).
3. Add 0.01% polyornithine solution to the wells on top of the cover slips. With inadequate volumes of polyornithine or laminin, the cover slips can float or remain uncoated. Per 6-well chamber, 2 mL polyornithine or laminin are used; 1 mL polyornithine or laminin is used for a 12-well chamber; 0.7 mL polyornithine or laminin is used per 24-well chamber (*see* **Note 11**).

3.3.2. Monolayer Neuronal Differentiation

The neurosphere protocol produces cultures in which more than 90% of the neurosphere clusters contain neuronal cells, but these cells are often located within large heterogeneous aggregates, making examination of individual cells and factors responsible for differentiation challenging. Alternative methods have been described that produce primarily neuronal cultures (greater than approx 80%), bypassing aggregation of cells in suspension culture *(**11**,**13**,**14**)*. Use of extremely low-density plating and genetic modification make two of these protocols difficult to apply to routine neuronal differentiation *(**11**,**13**)*.

Differentiation of ES cells in monolayer can be undertaken in two ways. In the first, a defined number of cells is plated onto gelatin-coated tissue culture plastic, and the ES cells are allowed to differentiate in N2:B27 medium for a desired time. In the second, ES cells are also initially plated onto gelatin but are then replated at specific densities onto polyornithine- and laminin-coated tissue culture plastic or cover slips in N2:B27 medium. The additional replating step produces a more uniform culture, results in a higher proportion of neurons, and depending on replating density, can produce a culture

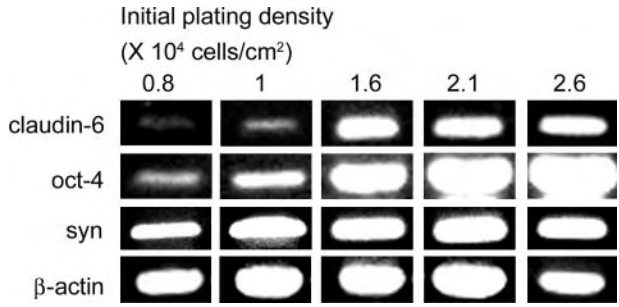

Fig. 2. Density-dependent expression of developmentally regulated transcripts. ES cell lines were differentiated for 14 d utilizing the monolayer differentiation protocol without replating at initial plating densities from 0.8 to 2.5 × 10^4 cells/cm^2. Semiquantitative RT-PCR was used to determine relative expression levels of a set of developmentally regulated transcripts. Claudin-6, a marker of epidermal ectoderm. Oct4, a POU transcription factor expressed in stem cells. Synaptophysin (Syn), a neuronal synaptic protein. β-Actin is employed as a loading control. As density increased, expression of the nonneuronal markers claudin-6 and Oct4 increased dramatically. Synaptophysin expression is robust at all densities.

with a density in which individual neurons or small clusters of neurons can be readily examined. The density of ES cells at initial plating and at replating has a profound impact on survival and the proportion of neurons obtained (*see* **Table 1**). At both the initial plating stage and at replating, the critical variable is the number of cells per unit surface area (square centimeter). We have explored the critical role of cell density in neuronal differentiation of murine ES cells without aggregation at both initial plating and at replating ***(18)***.

3.3.3. Optimal ES Cell Initial Plating Density for Neuronal Differentiation

Initial ES cell plating densities from 0.5 to approx 5 × 10^4 cells/cm^2 tissue culture plastic results in extensive neuronal differentiation (*see* **Fig. 1C,D**). At densities lower than 0.5 × 10^4 cells/cm^2, few cells survive. At plating densities of 0.8–1 × 10^4 cells/cm^2, a high proportion of cells (approx 50–70%) express the neuronal marker TUJ1 and have a neuronal morphology following 14 d of neuronal differentiation (*see* **Fig. 1C**). With initial plating densities greater than 1.5 × 10^4 cells/cm^2, robust neuronal differentiation is observed, but the cell density following 14 d of differentiation is too great to easily visualize individual cells, and the proportion of nonneuronal cells increases (*see* **Fig. 1D**). At higher plating densities, expression of nonneuronal markers also increases (*see* **Fig. 2**). The volume of medium utilized is dependent on well size.

An additional consideration for plating density is culture duration. When plating at densities of 0.8–1.0 × 10^4 cells/cm^2, by 48 h cells expressing neuronal precursor and neuronal markers are abundant. As culture proceeds, the number of cells expressing the neuronal marker TUJ1 increases, and cells with a mature neuronal morphology also increase rapidly in number. Between d 6 and 8, the number of cells with a mature neuronal morphology and the number of neuronal processes increase dramatically.

By d 14, neuronal cells have mature morphology (*see* **Fig. 1C**). With starting culture densities of greater than 1.0×10^4 cells/cm^2, following 14 d of neuronal differentiation without replating the cultures are very dense, and characterization of individual cells becomes more difficult (*see* **Fig. 1D**). We have maintained neuronal cultures for longer than 2 mo, but a large proportion of neuronal cells with a mature morphology are present by 14 d.

1. Day 1: Passage rapidly growing ES cells maintained in ES growth medium in T-25 flasks at 70–90% confluency 1:2 onto a new T-25 flask (approx 3.5×10^6 cells) for initiation of the monolayer neuronal differentiation protocol the following day.
2. Day 2: The T-25 flask should be 50–70% confluent. Remove cells from their substrate by the routine method (*see* **Subheading 3.1.**) and resuspend in a total volume of 2 mL N2:B27 medium (*see* **Note 12**).
3. Determine cell number (*see* **Subheading 3.2.2.**) and transfer the desired cell number per unit volume to gelatin-coated tissue culture plastic or into tissue culture wells into which cover slips have been placed and gelatin coated (*see* **Note 13**). To optimize cell adhesion, a total of 1.5 mL N2:B27 medium are used per well of a six-well plate for initial plating.
4. Gently exchange the medium every 2 d using 2 mL medium for a six-well tissue culture plate.

3.4. Optimal Replating Density and Timing for ES Cell Neuronal Differentiation

Although there is robust and primarily neuronal differentiation using the previously described protocol, an additional replating step can produce a more evenly distributed neuronal culture that is advantageous for some applications. Many of the same principles discussed also apply to this plating. At densities lower than 2.5×10^4 cells/cm^2, few cells survive. When cultures are continued for a total of 14 d, plating densities greater than 7.5×10^4 cells/cm^2 produce cultures that are too dense to easily characterize individual cells and become increasingly heterogeneous. As replating density increases to greater than 1.0×10^5 cells/cm^2, the proportion of nonneuronal cells rises. For a total culture length of 14 d, replating at $4–5 \times 10^4$ cells/cm^2 produces cultures with a uniform density that allows characterization of individual cells and small cellular clusters (*see* **Fig. 1E,F**). Compared to cultures that have not undergone replating, cultures replated at $4–5 \times 10^4$ cells/cm^2 are characterized by evenly distributed neuronal cells. Following replating, the cells take on a progressively more mature neuronal morphology; typically, between d 7 and 10 following the replating process, extension is robust. Cultures prepared in this method are 80–90% neuronal (*see* **Fig. 1E**).

1. On d 1, ES cell neuronal differentiation is initiated as in **Subheading 3.3.3.** at an initial plating density of $0.8–1.0 \times 10^4$ cells/cm^2 in a six-well, gelatin-coated tissue culture plate.
2. On differentiation d 4, wash each well of the six-well plate with 2 mL 1X HBSS and incubate with 0.7 mL 0.25% trypsin per well for 45–60 s. Gentle tapping of the plate facilitates release of the cells (*see* **Note 14**).
3. Remove cells to a 15-mL conical tube that contains 3 mL N2:B27 medium. Wash the well with an additional 1 mL N2:B27 medium and add it to the 15-mL conical tube (*see* **Note 15**).
4. Centrifuge the cellular suspension (2 min at a setting of 4 on the IEC centrifuge or equivalent approx 1500*g*). Remove the supernatant and resuspend the cell pellet in 2 mL N2:B27 medium to obtain a nearly single-cell suspension.

5. Count cells (*see* **Subheading 3.2.2.**) and replate at densities of 2.5×10^4 to 1.5×10^5 cells/cm^2 onto 6-, 12- or 24-well polyornithine- and laminin-treated tissue culture plastic or cover slips (*see* **Notes 16** and **17**). Once cells have been counted, the desired number of cells is placed into a total of 1.5 mL N2:B27 per six-well chamber to maintain a density of $4–5 \times 10^4$ cells/cm^2 of surface area (*see* **Note 18**).
6. Change the medium every 2 d. For a six-well plate, 2 mL N2:B27 are gently exchanged every 2 d.

3.5. Analysis of Differentiation

3.5.1. Immunohistochemistry

Many protocols exist for immunohistochemical staining of cells in culture. The following protocol works well with the primary antibodies raised against the neuronal marker neuronal class III tubulin and the POU transcription factor Oct4 expressed in ES cells.

1. On the desired d of differentiation, rinse cells in 1X PBS and fix in formaldehyde (3.7% in 1X PBS) for 10 min at room temperature on a rocking platform at approx 70 rpm.
2. Rinse with 1X PBS cells and store in 1X PBS at 4°C until required (*see* **Note 19**).
3. To begin the immunohistochemistry protocol, remove 1X PBS and permeabilize cells in 0.5% Triton X-100 for 10 min at on a rocking platform at 70 rpm at room temperature.
4. Rinse three times for 10 min each in 1X PBS on a rocking platform at room temperature.
5. Incubate in 1X PBS containing 10% serum for 20 min at room temperature on a rocking platform (*see* **Note 20**).
6. Incubate with primary antibodies at room temperature for 1 h on a rocking platform, 400 µL/well for a 6-well plate, 300 µL/well for a 12-well plate, and 200 µL/well for a 24-well plate (*see* **Note 21**).
7. Rinse three times in 1X PBS containing 0.1% Tween-20 at room temperature for 10 min.
8. Incubate with secondary antibodies at room temperature for 20 min (*see* **Note 22**).
9. Rinse three times in 1X PBS containing 0.1% Tween-20 at room temperature for 10 min.
10. Rinse with 1X PBS.
11. Incubate with Hoescht 33258 at 5 µg/mL (7.5 µL 10 mg/mL stock into 15 mL 1X PBS) for 2 min.
12. Rinse with 1X PBS.
13. Add 1X PBS and store in the dark wrapped in plastic wrap and aluminum foil at 4°C until viewed. If cover slips have been used, then they can be stored in 1X PBS prior to mounting onto glass slides.
14. To mount Thermanox cover slips on glass slides, carefully remove them from the 1X PBS with forceps and allow them to become nearly dry.
15. Mount cover slips in the following method to avoid refractive problems. For a 13-mm cover slip, place approx 35–50 µL mounting medium (i.e., gel/mount) onto the glass slide. Place the cover slip cell side up onto the mounting medium. Add 35–50 µL mounting medium directly to the cells on the cover slip. Place a 22-mm square glass cover slip on top of the Thermanox cover slip. Allow to dry in the dark overnight at room temperature (*see* **Note 23**).

3.5.2. Microscopy

Differentiated ES cells in six-well plates are viewed on an inverted scope. Cells differentiated on cover slips can be viewed on an upright microscope. Digital images are captured and analyzed using Adobe *Photoshop*.

Table 2
Thermocycling Parameters for Each Primer Pair

Gene (product size)	Primer sequences	Thermocycling parameters
Oct4 (541 bp)	5′-GATGGCATACTGTGGACCTCAG-3′ 5′-GCTTCGGGCACTTCAGAAAC-3′	(94°C/30 s, 55°C/1 min, 72°C/1 min) × 30, 72°C/7 min
Claudin-6 (287 bp)	5′-GATAGGAACTCCAAGTCTCGT-3′ 5′-TGGGACAGATGTAGAATAGCA-3′	94°C/3 min, (94°C/30 s, 51°C/1 min, 72°C/2 min) × 35, 72°C/2 min
Synaptophysin (287 bp)	5′-GCCTGTCTCCTTGAACACGAAC-3′ 5′-TACCGAGAGAACAACAAAGGGC-3′	94°C/3 min, (94°C/1 min, 60°C/1 min, 72°C/1 min) × 35, 72°C/7 min
β-Actin (405 bp)	5′-CAGGATTCCATACCCAAGAAGG-3′ 5′-AACCCTAAGGCCAACCGTG-3′	94°C/3 min, (94°C/30 s, 53°C/1 min, 72°C/1 min) × 30, 72°C/7 min

3.5.3. Reverse Transcription Polymerase Chain Reaction

1. Following differentiation, harvest RNA from the ES cell lines using Trizol reagent by the standard protocol.
2. Prior to reverse transcription with Superscript, treat the RNA pools with deoxyribonuclease I using the manufacturer's protocol.
3. Use 2 µg total RNA as the template for reverse transcription with Superscript primed with oligo dT 13mers performed by the standard protocol. Thermocycling parameters are optimized for each primer pair (*see* **Table 2**).
4. Following amplification, run products by electrophoresis on 1.3–2.0% agarose gels at 100–120 V.

Utilizing the monolayer neuronal differentiation protocol without replating as culture density increases, there is increased expression of the ES cell POU transcription factor Oct4 and the epithelial marker claudin-6. Expression of the neuronal marker synaptophysin is robust at all culture densities (*see* **Fig. 2**). The Notch/Delta signaling pathway is critical in the regulation of neuronal differentiation and likely plays a role in neuronal differentiation of ES cells ***(10)***. We found that as culture density increased, there was increased expression of the Notch ligands Jagged 1, Jagged 2, and Delta like 1; the Notch 1 receptor; and Notch effectors Hes 1 and 5.

4. Notes

1. Two neuronal differentiation media formulations, 80:20 and N2:B27, are utilized. Both are based on mixtures of N2 and B27 and provide a conducive environment for neuronal differentiation. They are based on previously published formulations with modification ***(14,17)***.
2. Retinoic acid has profound effects on ES cell differentiation and at differing concentrations may limit the range of neuronal subtypes produced ***(19)***.

3. The N2 and B27 supplements should not undergo repeated freeze/thaw cycles. The supplements should be aliquoted and thawed as needed. Both the 80:20 and N2:B27 medium formulations can be scaled to desired volumes but should be used within 2 wk of its preparation.
4. Make sure that there is no carryover of substrates into the culture medium as polyornithine can be toxic to ES cells, and soluble laminin can affect cell adhesion.
5. Neurospheres can be formed in volumes ranging from 6 to 20 mL as long as cell density is maintained at $4–6 \times 10^5$ cells/mL. Up to 8 mL can be used in a 60-mm dish, and up to 20 mL can be used in a 100-mm dish.
6. Counting and cell number calculation vary depending on a number of parameters and are carried out as described in the Coulter particle counter instruction manual. We utilize an aperture setting of 8.41 µm and 0.5-mL metered flow rate for ES cells. We add 20 µL of the cellular suspension to 20 mL IsotonII diluent (a 1000-fold dilution) and count five times. The highest and lowest values are discarded, and the remaining three counts are meaned. Because a 0.5-mL metered volume is used, the cell number per milliliter is obtained by multiplying the meaned value by 2000.
7. The critical variable in neurosphere production is the number of cells per volume of medium. The critical variable for plating of neurospheres or ES cells differentiated in monolayer is cell number per area of tissue culture plastic.
8. Occasionally, aggregates can become lightly attached to the tissue culture plastic; gentle tapping of the dish will release them. Residual adherent neurospheres can be removed by rinsing the plate with additional medium.
9. At this point in the protocol, ES cells are in neurospherelike aggregates, and it is not possible to obtain an accurate cell count. One way to estimate neurosphere plating density is to make calculations based on the initial number of cells used to create the neurospheres. As an example of this estimate, if neurospheres were formed in 9 mL medium at a density of 5×10^5 cells/mL, then the culture would initially contain 4.5×10^6 cells. To obtain a density of 5×10^5 neurosphere cells, the neurospheres would be resuspended in a total volume of 9 mL N2:B27 and 1 mL of the neurosphere suspension used per well of a six-well plate.
10. Cover slips are placed into the tissue culture dish with the tissue culture-treated side face up.
11. If cover slips float, they can be submerged with a pipet tip.
12. If ES cells do not adhere well to gelatin, adherence can be facilitated by using an equal volume of ES growth medium without LIF to inactivate trypsin and resuspension of the ES cell pellet in 2 mL ES growth medium without LIF.
13. The 6-, 12-, and 24-well plates can be utilized, provided that cellular number is adjusted for surface area. Glass cover slips can be utilized but have low adhesive properties. We routinely use 13-mm Thermanox tissue culture plastic cover slips. Up to four 13-mm cover slips conveniently fit into a chamber of a six-well plate. One 13-mm cover slip fits into either a 12- or a 24-well chamber. Plating onto Thermanox cover slips has the advantage that following differentiation cells can be imaged on an upright, confocal, or scanning electron microscope. Thermanox cover slips can also be embedded in resin and sections cut. Imaging of ES cells grown in tissue culture plastic dishes is primarily limited to use of an inverted scope. For initial plating, tissue culture plastic or tissue culture cover slips are prepared by covering the surface with 0.1% gelatin for 20 min to 24 h prior to use.
14. Differentiating ES cells can be replated on d 4–8, but because of the extensive neuronal processes present in culture by d 6, replating on d 4 may be optimal for most applications.
15. For some applications, it may be convenient to process three wells of a six-well plate at one time. In this case, 0.7 mL 0.25% trypsin is used per well. The 0.7 mL trypsin cellular suspension is collected from each well into a single 2-mL pipet, pooling the three wells into

approx 2.1 mL. This suspension is then used to gently rewash each well sequentially and then placed into a 15-mL conical tube containing 6 mL N2:B27 medium. An additional 3 mL N2:B27 is then used to wash residual cells from the three wells, and this is pooled with the other approx 8.1 mL, yielding a tube with approx 11.1 mL.

16. The Coulter particle counter aperture setting is decreased to 6.68 µm to more accurately count neuronal cells, which have a smaller diameter than undifferentiated ES cells.
17. At densities lower than 2.5×10^4 cells/cm^2, few cells survive. When cultures are continued for a total of 14 d, plating densities greater than 7.5×10^4 cells/cm^2 produce cultures that are too dense to easily characterize individual cells and become increasingly heterogeneous. As replating density increases to greater than 1.0×10^5 cells/cm^2, the proportion of nonneuronal cells rises. For a total culture length of 14 d, replating at 4–5 $\times 10^4$ cells/cm^2 produces cultures with a uniform density that allows characterization of individual cells and small cellular clusters (*see* **Fig. 1E,F**).
18. Cells can be replated onto 24-, 12-, or 6-well polyornithine- and laminin-treated tissue culture plastic as long as cell density is adjusted for well surface area. Cells can also be replated into tissue culture wells with polyornithine- and laminin-treated Thermanox cover slips. We routinely use 13-mm cover slips. Four 13-mm cover slips can be placed per chamber of a 6-well plate, and one can be placed into either a 12- or a 24-well plate. If cover slips are placed into tissue culture wells, then the total well surface area should be used for cell number calculation.
19. Individual cover slips can be removed to wells of a 12- or 24-well plate containing 1X PBS and stored at 4°C until they are stained.
20. The species of the serum should match that in which the secondary antibody was raised.
21. The murine monoclonal antibody against neuronal class III tubulin and goat polyclonal antibody against Oct4 can be mixed together in a single incubation.
22. The Cy3-conjugated affiniPure donkey antimouse and FITC-conjugated affiniPure donkey antigoat can be mixed together in a single incubation.
23. A variety of mounting media can be utilized, and it is recommended that manufacturer's instructions be followed.

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5

Generation of Inner Ear Cell Types From Embryonic Stem Cells

Marcelo N. Rivolta, Huawei Li, and Stefan Heller

Summary

The senses of hearing and balance are mediated by hair cells located in the cochlea and in the vestibular organs of the vertebrate inner ear. Loss of hair cells and other cell types of the inner ear results in hearing and balance disorders that substantially diminish the quality of life. The irreversibility of hearing loss in mammals is caused by the inability of the cochlea to replace lost hair cells. No drugs are available that stimulate inner ear cell regeneration. We describe here protocols to generate inner ear progenitor cells from murine ES cells and to differentiate these progenitors into hair cells and potentially into other inner ear cell types. In addition, we provide a modification of the protocol describing culture conditions in which human ES cells express a similar set of inner ear markers. Inner ear progenitor cells, generated from ES cells, may be used for the development of cell replacement therapy for the diseased inner ear, for high-throughput drug screening, and for the study of inner ear development.

Key Words: Auditory; cochlea; drug screening; hair cell; spiral ganglion; vestibular.

1. Introduction

The vertebrate inner ear is a fascinating organ that provides the sense of hearing, balance, and the ability to detect acceleration forces. All these mechanical stimuli are mediated by hair cells located in the mammalian cochlear organ of Corti, in the two vestibular epithelia of the utricle and saccule, and in the three ampullae at the bases of the semicircular canals. Hair cells are named for their stereociliary bundles, which protrude from the cells' apical surfaces. Hair bundles mediate mechanosensitivity, and their highly organized structure plays critical roles in mechanoelectrical transduction and force amplification. Hair cells are an extremely rare cell type; approx 14,000 hair cells (approx 3500 "inner" hair cells and approx 12,000 "outer" hair cells), intertwined with different types of supporting cells, can be found in the mammalian cochlea *(1)*.

Albeit hair cells often play the leading part, many other cell types are essential for proper cochlear and inner ear function. Auditory and vestibular ganglion cells provide afferent innervation to hair cells and send axonal projections into the central nervous

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system. Various types of fibrocytes of the spiral ligament and the ion-transporting cell types of the stria vascularis are also essential cochlear components.

The paucity of sensory cells in the inner ear and the complexity of the organ make them difficult to isolate and thus challenging to purify cellular material for applied molecular-biological and biochemical studies. Consequently, not many molecular-biological tools exist for inner ear cell types *(2)*. As a result, understanding of hair cell mechanosensation at the molecular level is just beginning. This limitation was one of the driving forces that inspired the development of a protocol allowing the generation of inner ear cell types, particularly hair cells, from a renewable and abundant source: embryonic stem (ES) cells *(3)*.

Two equally important and interesting applications for a protocol to differentiate inner ear cell types from ES cells are the potential clinical applications to treat inner ear disorders and the use of in vitro-generated otic progenitor cells to study inner ear development. Underlying the irreversibility of hearing loss in mammals is the incapacity to replace lost hair cells by cell division or by regeneration from endogenous cells *(4)*. Hair cell replacement, either by stimulation of regeneration (as occurs naturally in non-mammalian vertebrates) or by transplantation of progenitor cells capable of differentiating into hair cells, remains therefore the ultimate goal in the development of treatment applications to reconstruct the damaged inner ear *(5)*. Stem cell-derived progenitor cells are an exquisite vantage point either for transplantation studies into damaged inner ears or for high-throughput assays to identify drugs or drug targets that modulate hair cell differentiation. The results of such screens may provide novel drugs for treatment of hearing loss, and the genes identified in such assays will be important candidates for controlling inner ear development.

ES cells have been shown to differentiate into various cell types, particularly into ectodermal derivatives *(6,7)*. We describe protocols here for a stepwise approach to first generate inner ear progenitors from murine ES cells and then to differentiate these progenitors into hair cells and potentially into other inner ear cell types. In addition, we provide a modification of the protocol describing culture conditions in which human ES (hES) cells express a similar set of inner ear markers. This is a reproducible, powerful basic protocol for the creation of inner ear progenitors that can be differentiated in vitro or in vivo into hair cells *(3)*.

2. Materials

2.1. Cell Culture

2.1.1. Cell Culture Equipment

1. Dedicated room or work area (*see* **Note 1**).
2. Biosafety cabinet or laminar flow hood.
3. Refrigerator (4°C) and freezer (–20°C).
4. Humidified incubator(s) at 37°C with 5% CO_2 and 95% air atmosphere.
5. Inverted microscope with ×5, ×10, and ×20 phase contrast lenses.
6. Hemocytometer.
7. Pipetman-20, -200, and -1000.
8. BD Falcon 15-mL and 50-mL sterile conical tubes (BD Biosciences, San Jose, CA; cat. no. 352097 and 352098, respectively).

9. BD Falcon 5-, 10-, and 25-mL sterile plastic pipets (BD Biosciences, cat. no. 357551, 357543, and 357525, respectively).
10. Pipet aid (Integra Pipetboy acu, Argos Technologies, East Dundee, IL; cat. no. 155000).
11. BD Falcon 100-mm cell culture dishes, standard tissue-culture (TC) (BD Biosciences, cat. no. 353003).
12. 35-mm, four-well, standard TC cell culture dishes (Greiner Bio-One, Longwood, FL; cat. no. 627170).
13. T-25 and T-75 cell culture flasks (Nunc, Rochester, NY; cat. no. 178891).
14. 60- and 100-mm bacterial Petri dishes (Bibby Sterilin, Staffordshire, England; cat. no. 123; Kord-Valmark, Brampton, Canada; cat. no. 900).
15. CryoTube cryogenic storage vials (Nunc cat. no. 377224).
16. Nalgene Cryo 1°C freezing container (Nalgene, Rochester, NY; cat. no. cryoware 5001).

2.1.2. Media, Supplements, Solutions, and Other Supplies

1. 100 mL phosphate-buffered saline (PBS) at pH 7.2 (1X; Invitrogen, Carlsbad, CA; cat. no. 20012-027).
2. High-glucose Dulbecco's modified Eagle's medium (DMEM) with GlutaMax, 4.5 g/L D-glucose, and sodium pyruvate (500 mL; Invitrogen, cat. no. 10569-010).
3. Knockout™ DMEM (500 mL; Invitrogen, cat. no. 10829-018).
4. Knockout serum replacement (500 mL; Invitrogen, cat. no. 10828-028).
5. Qualified fetal bovine serum (FBS) (500 mL; Invitrogen, cat. no. 10437-028) and ES cell-qualified FBS (500 mL; Invitrogen, cat. no. 10439-024) (*see* **Notes 2** and **3**).
6. 100 mL 10 m*M* minimum essential medium (MEM) nonessential amino acids (100X; Invitrogen, cat. no. 11140-050).
7. 50 mL 55 m*M* β-mercaptoethanol (β-ME) (1000X liquid; Invitrogen, cat. no. 21985-023).
8. 100 mL 200 m*M* L-glutamine (100X; Invitrogen, cat. no. 25030-081).
9. 20 mL 10-mg/mL ampicillin sodium salt solution (100X; Sigma, St. Louis, MO; cat. no. A0797).
10. 100 mL penicillin-streptomycin: 10,000 U penicillin and 10 mg streptomycin/milliliter (100X; Invitrogen, cat. no. 15140-122).
11. 1 mL leukemia inhibitory factor (LIF), 10^6 U/mL (100X; Rat ESGRO, Chemicon, Temecula, CA; cat. no. ESG1106).
12. 500 mL ES cell-qualified 0.1% gelatin (Specialty Media, Phillipsburg, NJ; cat. no. ES-006-B).
13. 100 mL trypsin-ethylenediaminetetraacetic acid (EDTA): 0.25% trypsin, 1 m*M* EDTA • 4Na (1X; Invitrogen, cat. no. 25200-056).
14. Complete DMEM: high-glucose DMEM supplemented with 10% FBS, 1X penicillin-streptomycin.
15. Complete mouse ES cell medium: for 575 mL, add 75 mL ES cell-qualified FBS to 500 mL knockout DMEM and mix. Then, add 5.75 mL 100X MEM nonessential amino acids, 575 µL 1000X β-ME, 5.75 mL 100X *L*-glutamine, and 5.75 mL 100X ampicillin.
16. Mitomycin C solution: resuspend the contents of a 2-mg vial mitomycin C (Sigma, cat. no. M4287) in 5 mL H_2O, filter-sterilize, and store in 125-µL aliquots at –20°C.
17. Mitomycin C medium: add 125 µL mitomycin C solution to 5 mL complete DMEM; warm to 37°C and use immediately.
18. 50 mL cell culture freezing medium (Invitrogen, cat. no. 11101-011).
19. 50 mL ES cell-qualified cell culture freezing medium (2X; Specialty Media, cat. no. ES-002-D).
20. 500 mL Hanks balanced salt solution (HBSS) (1X; Invitrogen, cat. no. 14025-092).
21. 1 g collagenase IV (Invitrogen, cat. no. 17104-019).

22. Collagenase IV solution: dissolve 1 g collagenase IV in 250 mL HBSS, filter-sterilize, and store in 5- to 10-mL aliquots at –20°C.
23. Complete hES medium: for 250 mL, mix 200 mL knockout DMEM with 50 mL Knockout serum replacement. Add 2.5 mL 100X MEM nonessential amino acids solution, 1.25 mL 200 m*M* L-glutamine, 0.5 mL 55 m*M* β-ME, and 50 µL 50 µg/mL basic fibroblast growth factor (bFGF) stock solution. Store at 4°C and use within 2 wk.
24. 1.5-mL nonstick microfuge tubes (pack of 250; Ambion, Austin, TX; cat. no. 12450).
25. 5 mL N2 cell culture supplement (100X; Invitrogen, cat. no. 17502-048).
26. 200 µg recombinant human epidermal growth factor (EGF) (R&D Systems, Minneapolis, MN; cat. no. 236-EG). To generate a 1000X stock solution (20 µg/mL), reconstitute the content of the vial in 10 mL Knockout DMEM, sterile filter, and store in 100-µL aliquots at –20°C.
27. 50 µg recombinant mouse insulinlike growth factor (IGF) 1 (R&D Systems, cat. no. 791-MG-050). To generate a 1000X stock solution (50 µg/mL), reconstitute the content of the vial in 1 mL Knockout DMEM, sterile filter, and store in 50-µL aliquots at –20°C.
28. 25 µg recombinant human bFGF (R&D Systems, cat. no. 234-FSE). To generate a 500X stock solution (50 µg/mL), reconstitute the content of the vial in 0.5 mL Knockout DMEM, sterile filter, and store in 50-µL aliquots at –20°C.
29. 1 g purified trypsin inhibitor from soybean (Worthington, Lakewood, NJ; cat. no. LS003571). To generate a 100X stock solution (250 mg/mL), reconstitute the content of the vial in 4 mL PBS, sterile filter, and store in 50-µL aliquots at –20°C.
30. 100 mL 0.1 mg/mL poly-L-ornithine solution (Sigma, cat. P4957).

2.2. Semiquantitative Reverse Transcription Polymerase Chain Reaction

1. RNeasy midikit (midikit [10], Qiagen, Valencia, CA; cat. no. 75142).
2. 250 mL RNaseZap (Ambion, cat. no. 9780).
3. 100 mL 14.3 *M* pure liquid β-ME (Sigma, cat. no. M7522).
4. Buffer RLT-β-ME: buffer RLT is supplied with the RNeasy midikit. Add 40 µL β-ME to 4 mL buffer RLT (*see* **Note 4**).
5. 21-gage needle (BD Biosciences, cat. no. 305165).
6. 5-mL syringe (BD Biosciences, cat. no. 309603).
7. 500 mL RNase-free water (Ambion, cat. no. 9920).
8. 1 pint 200-proof ethanol (Pharmco Products, Brookfield, CT; cat. no. 111ACS200).
9. 90% ethanol: mix from 200-proof ethanol and RNase-free water.
10. Ultraviolet spectrophotometer with microquartz cuvet (e.g., Bio-Rad SmartSpec 3000).
11. 100 mL 3 *M* sodium acetate at pH 5.5 (Ambion, cat. no. 9740).
12. 500 mL isopropanol (Sigma, cat. no. I9516).
13. Speed Vac (e.g., Savant DNA 110).
14. 25 µg Oligo$(dT)_{12–18}$ (Invitrogen, cat. no. 18418-012).
15. Oligo(dT) mix: dissolve 25 µg oligo$(dT)_{12–18}$ in 550 µL RNase-free water; store in 50-µL aliquots at –20°C.
16. 100 µL 10 m*M* dNTP mix (Invitrogen, cat. no. 18427-013).
17. SuperScript II reverse transcriptase, including 5X first-strand buffer and 0.1 *M* dithiothreitol solution (2000 U; Invitrogen, cat. no. 18064-022).
18. RNase H (30 U; Invitrogen, cat. no. 18021-014).
19. 100 mL 1 *M* Tris-HCl at pH 7.0 (Ambion, cat. no. 9850G).
20. 10 m*M* Tris-HCl at pH 7.0: mix 0.5 mL 1 *M* Tris-HCl at pH 7.0 with 49.5 mL MilliQ water; autoclave.

21. Thermal cycler (e.g., GeneAmp PCR system 9700, Applied Biosystems, Foster City, CA).
22. *Taq* DNA polymerase (500 U; Promega, Madison, WI; cat. no. M1665).
23. 750 µL *Taq* DNA polymerase 10X reaction buffer with $MgCl_2$ (Promega, cat. no. M1881).
24. 100 mL dimethyl sulfoxide (DMSO) (Sigma, cat. no. D8418).
25. 0.2-mL thin-wall polymerase chain reaction (PCR) reaction tubes (USA Scientific, Ocala, FL; cat. no. 1402-4300).

2.3. Immunofluorescence

1. 16% paraformaldehyde solution (10-mL ampules [10]; Electron Microscopy Sciences, Hatfield, PA; cat. no. 15710): dilute 1:4 in PBS to generate a 4% paraformaldehyde solution.
2. 250 mL Triton X-100 (Fisher Scientific, Fairlawn, NJ; cat. no. AC21568-2500).
3. 100 g bovine serum albumin (BSA) (Fisher Scientific, cat. no. FLBP1600-100).
4. 100 mL goat serum (Invitrogen, cat. no. 16210-064): heat inactivate for 30 min at 56°C; store frozen at –20°C in 10-mL aliquots.
5. PBT1: 0.1% Triton (v/v), 1% BSA (w/v), and 5% (w/v) heat-inactivated goat serum in PBS. Prepare 500 mL and store at –20°C in 25-mL aliquots.
6. PBT2: 0.1% Triton (v/v) and 0.1% BSA (w/v) in PBS. Prepare 500 mL and store at –20°C in 25-mL aliquots.
7. 10 mL Vectashield mounting medium (Vectorlabs, Burlingame, CA; cat. no. H-1000).
8. 10 mL Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vectorlabs, cat. no. H-1200).
9. 10 mg DAPI (Sigma, cat. no. D9542): prepare a stock of 10 mg/mL in water and store frozen and protected from light in small aliquots.
10. 10-mm diameter round cover slips (no. 1, Menzel-Glaeser, Braunschweig, Germany; cat. no. CB00100RA1).

3. Methods

3.1. Culture of Undifferentiated Mouse ES Cells

Mouse ES cells can be maintained undifferentiated by growing them on feeder layers of mitotically inactivated fibroblasts or on gelatin-coated culture dishes in the presence of LIF (*see* **Fig. 1A**,**B**); here, both methods are described. We always use inactivated fibroblasts for the culture of freshly thawed ES cells and for general ES cell maintenance. Mouse ES cells to be used for in vitro differentiation are usually weaned for one or two subculture periods to gelatin-coated dishes.

3.1.1. Preparation of Mouse Embryonic Fibroblasts

1. Remove, under sterile conditions, 2–10 E13.5 embryos from the embryonic sac following local ethical guidelines and regulations.
2. Decapitate and eviscerate the embryos, wash carcasses with PBS, and mince with scalpel blade.
3. Transfer the minced tissue into a 50-mL conical tube, add PBS to 40 mL, close the cap and shake carefully; let the tissue pieces settle for 1 min and carefully aspirate the PBS.
4. Add 2 mL trypsin-EDTA per embryo and incubate at 37°C for 15 min in a shaking water bath (*see* **Note 5**).
5. Inactivate trypsin by adding 20 mL complete DMEM, mix thoroughly, and carefully break up clumps by pipetting with a 25- or 50-mL plastic pipet.
6. Let tube sit on a rack for 3–5 min to allow tissue clumps to settle by gravity. Carefully transfer supernatant with the suspended cells to a fresh tube.

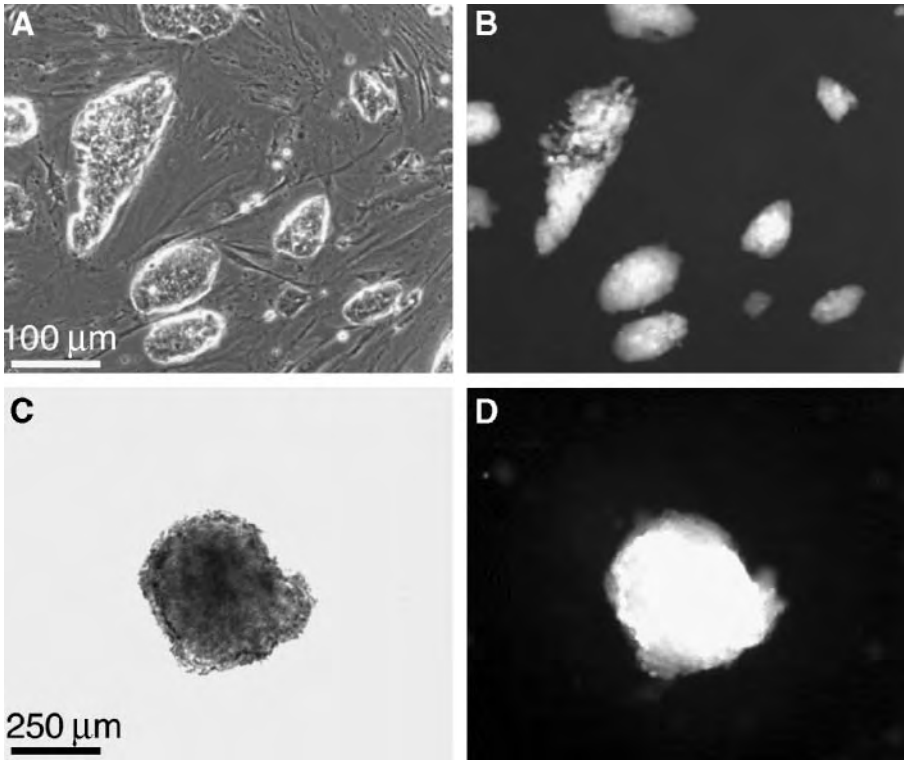

Fig. 1. Murine embryonic stem (ES) cells and embryoid bodies. (**A**) ES cells growing in dense clusters on a mitotically inactivated mouse embryonic fibroblasts feeder layer. (**B**) This particular line of ES cells expresses a fluorescent protein under control of a ubiquitous promoter *(12)*, which allows distinguishing between the fluorescent ES cell clusters and the nonfluorescent feeder cells. (**C**) Typical embryoid bodies (EBs) formed after 7 d of culture in hanging drops. (**D**) Fluorescence of the EBs. Using a marker protein can be helpful in subsequent experiments, for example, to detect the stem cell derivatives after cell transplantation into a host animal *(3,13–15)*.

7. Collect the cells by centrifugation at 200*g* for 5 min and carefully aspirate the supernatant without disturbing the cell pellet.
8. Resuspend in 20 mL complete DMEM and split into T-75 cell culture flasks. Use the equivalent of cells of two embryos per flask and add complete DMEM for a total volume of 25 mL in each flask. Grow in a humidified incubator until approx 90% confluency, which usually takes 1–3 d, and freeze 15 750-µL stock vials per T-75 culture (*see* **Subheading 3.1.4.**). These frozen stocks are considered passage zero (P0) mouse embryonic fibroblasts (MEFs).

3.1.2. Maintenance of MEF Cultures and Mitotic Inactivation of MEFs

MEFs are subcultured on confluency by splitting them every third or fourth day (e.g., Mondays and Fridays) at a 1:3 to 1:4 ratio. Do not use MEFs beyond passage six.

1. Aspirate the medium from a confluent 100-mm MEF culture, wash the cells with 5 mL PBS at room temperature, and replace the PBS with 1 mL trypsin-EDTA. Tilt to cover the whole

surface and incubate for 2–3 min at 37°C or longer until the cells detach by gentle tapping of the culture dish.
2. Add 5 mL complete DMEM and break up cell clumps by gentle pipetting with a 10-mL plastic pipet.
3. Fill a fresh 100-mm culture dish with 9 mL complete DMEM.
4. Dilute 1.5–2 mL MEF cell suspension into the new culture dish with fresh medium. Do not forget to label the dish to keep track of the passage number.
5. Maintenance and preparation of mitotically inactivated MEFs can be done in parallel by expanding MEFs into several 100-mm dishes.
6. For maintenance, grow cells to confluency, which should be reached within 3–4 d. Adjust dilution of the cells when needed.
7. For inactivation, grow cells to confluency and replace medium carefully with 5 mL warm (room temperature, 37°C) mitomycin C medium.
8. Incubate at 37°C for 3 h.
9. Meanwhile, prepare one to four 100-mm culture dishes by adding 5 mL 0.1% gelatin and let the dishes sit for 1–2 h at room temperature to gelatin coat the surfaces.
10. Aspirate mitomycin C medium, wash three times with PBS, and trypsinize the cells with 1 mL trypsin-EDTA for 2–3 min at room temperature or longer until the cells detach by gentle tapping of the culture dish.
11. Transfer the cells to a 15-mL conical tube containing 9 mL complete DMEM and spin down at 200*g* for 5 min.
12. Resuspend the pelleted cells in 10 mL complete DMEM.
13. Remove the gelatin solution from the 100-mm dish(es).
14. Plate 2–3 mL of the inactivated MEF suspension per 100-mm dish in a total volume of 10 mL complete DMEM (*see* **Note 6**).
15. Incubate the seeded inactivated MEFs overnight at 37°C to settle. Use the feeders within 1 wk.

3.1.3. Thawing Cryopreserved Cells

1. For thawing ES cells, have a 60-mm dish with mitogenically inactivated MEFs ready (*see* **Subheading 3.1.2.**). Selected progenitors (*see* **Subheading 3.3.5.**) are seeded onto 60- or 100-mm poly-L-ornithine-/gelatin-coated dishes, and MEFs will do well on regular 100-mm cell culture dishes.
2. Fill a 15-mL conical tube with 10 mL complete growth medium (complete ES cell medium or complete DMEM).
3. Remove vial from liquid nitrogen storage and quickly thaw in a 37°C water bath.
4. Wipe the outside of the vial extensively with 70% ethanol.
5. Gently and slowly transfer the 0.5–1.0 mL thawed cell suspension into the 10 mL of complete growth medium and gently mix.
6. Pellet the cells at 200*g* for 5 min and aspirate the medium carefully without disturbing the cells.
7. Gently resuspend the cells in complete growth medium and plate.
8. Change medium after 12–14 h (*see* **Note 7**).

3.1.4. Cryopreservation of Cells

MEFs, ES cells, and selected progenitors are frozen with a standard cell freezing protocol that can also be used to freeze other vertebrate cell lines. Cell that are cryopreserved can be stored for a few months at –80°C or indefinitely at –196°C in liquid nitrogen. To maintain high viability, it is recommended to store all frozen cells in

liquid nitrogen. Cells that are going to be frozen should be harvested in the log phase of growth, before they reach confluency.

1. Thaw cell culture freezing medium (*see* **Note 8**, when freezing ES cells), mix well, and keep on ice until use.
2. Detach the 70–80% confluent cells grown in 100-mm dishes with the appropriate enzymatic treatment (*see* **Note 9**).
3. Stop the enzymatic treatment with 5 mL complete ES medium and transfer the cells into a 15-mL conical tube.
4. Pellet the cells at 200*g* for 5 min and aspirate the medium carefully without disturbing the cells.
5. Resuspend the cells in 5 mL ice-cold cell culture freezing medium (*see* **Notes 8** and **10**).
6. Aliquot into cryogenic storage vials, label vials, and keep on ice. Freezing the cells should commence within 10 min.
7. Transfer the vials into a cooled (4°C) freezing container and place the freezing container immediately into a –80°C freezer for 24 h (*see* **Note 11**).
8. Transfer the vials into a liquid nitrogen cryo storage container for long-term preservation.

3.1.5. Maintenance of Undifferentiated Mouse ES Cells on Feeder Layers

Feeder layers of mitotically inactivated MEFs are used for regular ES cell maintenance and to recover ES cells from thawing (*see* **Fig. 1A**,**B**). ES cells are never cultured for extended time periods as they begin to differentiate and accumulate cells with abnormal karyotypes. As a general rule, ES cells are immediately expanded after isolation from blastocysts or on receipt from another laboratory and frozen after as few passages as possible. Nevertheless, it is also important to ensure that as many viable aliquots as possible are generated when freezing low passage number ES cells. Adequate expansion is usually obtained after two or three passages.

1. Compose a time schedule for the complete culture period of ES cells and ensure to maintain, in parallel, low passage number MEFs (*see* **Subheadings 3.1.1.** and **3.1.2.**).
2. Prepare an adequate number of mitogenically inactivated MEFs (*see* **Subheading 3.1.2.**).
3. Replace 7 mL medium from each inactivated MEF 100-mm culture dish with 7 mL complete mouse ES medium (*see* **Note 12**).
4. Wash the ES cells growing at medium-high density in a 100-mm culture dish with 5 mL PBS.
5. Add 1 mL 1X trypsin-EDTA. Tilt to cover the whole surface and incubate for 2–3 min at room temperature.
6. Check under a microscope to ensure cells have detached from the surface and are dissociating.
7. Add 5 mL complete mouse ES medium and break up cell clumps by carefully pipetting up and down and rinsing the entire surface area of the dish.
8. Transfer to a 15-mL conical tube and spin down at 200*g* for 5 min.
9. Resuspend the pelleted cells in 5 mL complete mouse ES medium.
10. Seed 0.5–0.7 mL (or approx 3×10^6 cells; *see* **Note 13**) onto the inactivated MEF feeders. Supplement the total volume (approx 10 mL) of complete mouse ES medium with 1000 U LIF per milliliter.
11. Distribute the cells evenly when placing the dish into the incubator.

ES cells grow in dense clusters on top of the feeders (*see* **Fig. 1A**,**B**) and are passed twice per week. Of the exhausted media, 75% is usually changed every other day (*see* **Note 14**).

3.1.6. Maintenance of Undifferentiated Mouse ES Cells on Gelatin-Coated Dishes

1. At 1–2 h before passing the ES cells, prepare a 100-mm culture dish (or more) by adding 5 mL 0.1% gelatin and incubate at room temperature to coat the surface of the dish.
2. Wash the ES cells growing at medium-high density in a 100-mm culture dish with 5 mL PBS.
3. Add 1 mL 1X trypsin-EDTA. Tilt to cover the whole surface and incubate for 2–3 min at room temperature.
4. Check under a microscope to ensure that cells have detached from the surface and are dissociating.
5. Add 5 mL complete mouse ES medium and break up cell clumps by carefully pipetting up and down and rinsing the entire surface area of the dish.
6. Transfer to a 15-mL conical tube and spin down at 200*g* for 5 min.
7. Resuspend the pelleted cells in 5 mL complete mouse ES medium.
8. Seed 0.5–0.7 mL (or approx 3×10^6 cells; *see* **Note 13**) in 10 mL complete mouse ES medium supplemented with 1000 U LIF per milliliter.
9. Check cells daily and replace the exhausted medium every other day or more often, if necessary. Pass cells every 3–4 d before the islands of ES cells start growing into each other.

3.2. Culture of Undifferentiated hES Cells

Induction of molecular markers for inner ear cell types can also be achieved with hES cells using a slightly modified protocol of the one used to generate inner ear cell types from mouse ES cells. For comparison, hES cell culture and guidance protocols are presented next.

Unlike mouse ES cells, the maintenance of undifferentiated hES cells is not facilitated by LIF. hES cells therefore require the presence of a layer of feeder MEFs. Human undifferentiated ES cells do not tolerate suspension well as single cells generated by trypsin treatment. It is therefore recommended to detach colonies by digestion with collagenase and to maintain the hES cells as aggregates.

1. Compose a time schedule for the complete culture period of hES cells and ensure to maintain, in parallel, low passage number MEFs (*see* **Subheadings 3.1.1.** and **3.1.2.**).
2. Aspirate the medium from a confluent 100-mm hES cell culture, wash the cells with 5 mL PBS, and replace the PBS with 2 mL collagenase IV solution.
3. Tilt dish to cover the whole surface and incubate for 5–8 min at 37°C. Monitor microscopically every 2 min until the edges of the colonies begin to curl.
4. Aspirate collagenase and replace with 5 mL hES medium.
5. Gently loosen the cells in clumps with a 10-mL pipet (*see* **Note 15**).
6. Paying attention not to dissociate the cells completely to single cells, gently pipet the solution up and down and then transfer the medium with the cell clumps to a 15-mL conical tube.
7. Spin at 50*g* for 3 min.
8. Aspirate the supernatant from the hES cell pellet and very gently resuspend the cells, maintaining clumps, in 5 mL hES medium.

9. Remove the medium from a 100-mm dish of inactivated MEF feeders and wash the feeders gently once with 10 mL PBS, then replace the PBS with 9 mL hES medium.
10. Seed 1.2–1.5 mL resuspended hES cells per 100-mm feeder cell dish (*see* **Note 16**). Carefully place in incubator, making sure that the cells are evenly distributed across the dish.

3.3. Generation of Inner Ear Progenitor Cells

The inner ear is derived from an ectodermal thickening near the developing hindbrain. This otic placode invaginates to form the otic cup, which pinches off to form the otic vesicle. The majority of inner ear cell types are derived from the otic vesicle, including hair cells as well as auditory and vestibular neurons. Cells of the otic placode and cup are highly proliferative and express the paired-box transcription factor Pax-2 ***(8)***.

We consequently define potential inner ear progenitor cells as proliferative and Pax-2 positive, boldly aware of the fact that other progenitors (e.g., for the kidney) also display these features. We further hypothesized that growth factors that have been shown to mitogenically or trophically support inner ear progenitors would suffice in promoting survival of Pax-2-positive progenitor cells from a mixed ES cell-derived population when cultured in absence of serum and additional mitogens.

This process is done stepwise. First, embryoid bodies (EBs), spherical structures that contain early cell types of all three germ layers, are generated from ES cells ***(9)*** (*see* **Fig. 1C,D**). Second, EB-derived cells are then cultured for an extended period of time in serum-free medium in the presence of EGF and IGF-1. Third, the selectively enriched cell population is expanded by addition of the mitogenic bFGF. At this point, selected inner ear progenitors are stable for five to seven passages (*see* **Fig. 2A–C**); nevertheless, they will undergo a shift in their capability to generate various cell types at the cost of their potency to generate hair cells. Finally, the selected inner ear progenitors can be differentiated by withdrawal of all growth factors, and mature inner ear cell types can be identified (*see* **Fig. 2D–H** and **Fig. 3**).

3.3.1. EB Formation in Hanging Drop Cultures

EBs are generated by aggregation of ES cells. Differentiating ES cells will form cell types of all three germ layers, and individual progenitor cell types can subsequently be enriched by many different methods. It appears that the size and the culture period of the initial cell aggregates are important for a homogeneous population of EBs (*see* **Fig. 1C,D**). To ensure this, EBs for inner ear progenitor cell generation are formed in hanging drops for a period of 6–8 d.

3.3.2. Murine EB Formation in Hanging Drop Cultures

1. Dissociate mouse ES cells as described in **Subheading 3.1.6.**
2. Count cells and dilute 5×10^4 undifferentiated ES cells in 5 mL DMEM with 15% FBS (without LIF).
3. Lay 30–50 30-µL drops (at this dilution, each drop will contain approx 300 cells) on the underside of the lid of a 150-mm plastic Petri dish.
4. Fill the Petri dish with 15 mL PBS.
5. Lift lid gently and invert it, taking care not to dislodge the drops; place on top of the dish containing the PBS (*see* **Note 17**).
6. Incubate at 37°C for 3–4 d.

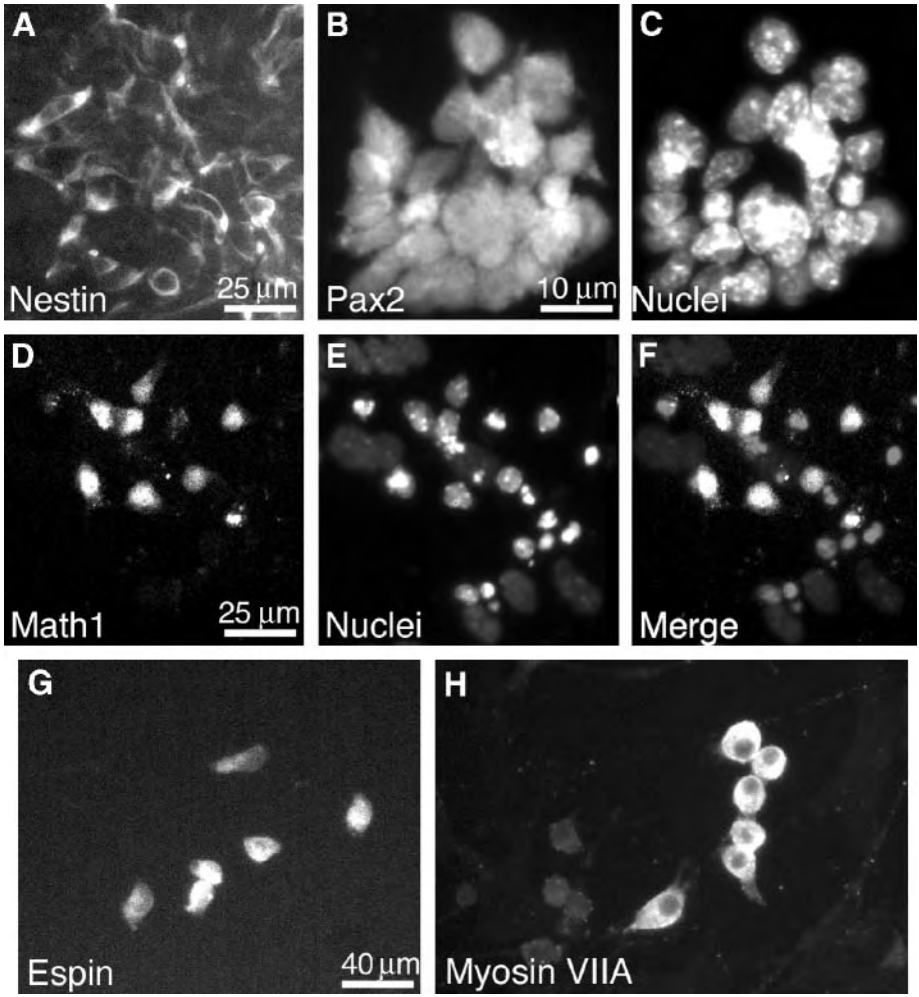

Fig. 2. Selected murine inner ear progenitors and differentiated cells. (**A**) The majority of selected inner ear progenitors can be labeled with antibody to nestin. (**B**) Pax2-positive inner ear progenitors cells. (**C**) Nuclear staining of the cells shown in (**B**) with the nuclear marker 4′,6-diamidino-2-phenylindole (DAPI). (**D**) Differentiated cells express the hair cell marker Math1, here visualized with nuclear fluorescence with antibody to the transcription factor. (**E**) Nuclear staining of the cells shown in (**D**) with the nuclear marker DAPI. (**F**) The merged image visualizes that only a subpopulation of the differentiated cells express Math1. (**G**) A subpopulation of the differentiated cells expresses the hair cell marker espin. (**H**) Myosin VIIA expression by a subpopulation of the differentiated cells.

3.3.3. Human EB Formation

Human ES cells do not perform well in the production of EBs when following the hanging drop method. The recommended method is to allow their formation on

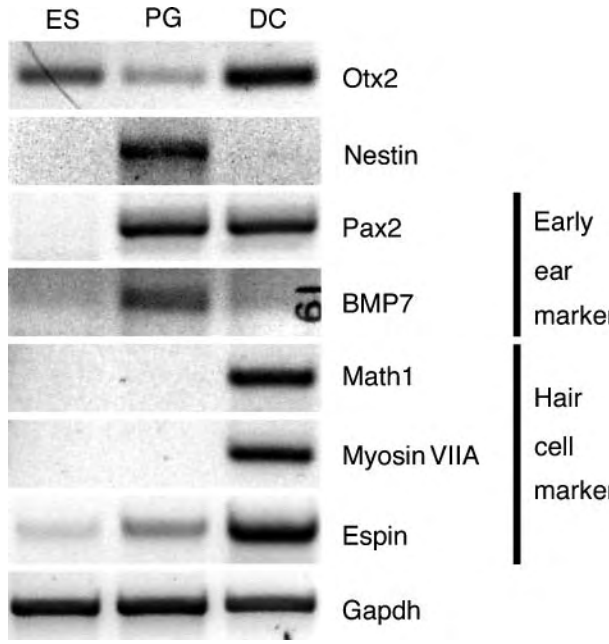

Fig. 3. Comparative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of marker expression by embryonic stem (ES) cells, selectively enriched inner ear progenitors (PG), and cells after in vitro differentiation (DC). Genes that are indicative of early inner ear and developing inner ear sensory epithelia are designated with "early ear marker." Transcripts for these genes, such as Pax2 and BMP7, are upregulated after progenitor cell selection. RT-PCR products for hair cell markers—shown here are Math1, myosin VIIA, and espin—are substantially upregulated in the differentiated cell population.

untreated Petri dishes. The nonadherent nature of the microbiology-grade surfaces prevents the attachment of the EBs.

1. Dissociate hES cells as described in **Subheading 3.2.**
2. Transfer the detached colonies to a 15-mL conical tube.
3. Spin at 50*g* for 3 min.
4. Aspirate the supernatant from the hES cell pellet and very gently resuspend the cells, maintaining clumps, in hES medium (without bFGF) supplemented with 20 ng/mL EGF and 50 ng/mL IGF-1.
5. Transfer to a sterile 60-mm bacterial Petri dish. Six to nine plates can be set up from a 100-mm dish of undifferentiated hES cells using 3 mL culture media per 60-mm dish.
6. Feeding is done every other day by transferring the EBs with their culture medium and allowing them to settle under gravity (takes 5–10 min). The old medium is then aspirated, and new medium with fresh growth factors is added, taking care not to disrupt the spheres. The whole preparation is then transferred to a fresh bacterial Petri dish.
7. EBs are grown for 4–6 d.

3.3.4. Selective Enrichment for Inner Ear Progenitor Cells

1. Generate 50 murine or human EBs as described in **Subheadings 3.3.2.** and **3.3.3.**
2. At 1–2 h before harvesting the EBs, take five 100-mm poly-L-ornithine-coated culture dishes (*see* **Note 18**), add 5 mL 0.1% gelatin to each dish, and incubate at room temperature to coat the surface of the dish with gelatin.
3. Prepare five 1.5-mL nonstick sterile microfuge tubes by filling them with 1 mL PBS.
4. Using a P-1000 pipet, gently collect 10 EBs and transfer them into one of the prepared tubes. Repeat until all five tubes have received 10 EBs each.
5. Spin at 100*g* for 3 min.
6. Carefully remove the supernatant and add 150 µL 0.5X trypsin-EDTA into each tube. Flick the bottom of each tube to resuspend the EBs and incubate for 5 min at 37°C in a shaking water bath (*see* **Note 5**).
7. Add 200 µL complete DMEM medium.
8. With a P-200 pipet, carefully triturate the trypsinized EBs by pipetting up and down 10–20 times.
9. Remove 10 µL cell suspension and check under the microscope to ensure that the cells are dissociated.
10. Replace the gelatin solution in the five 100-mm culture dishes with 10 mL high-glucose DMEM medium with 15% FBS and 100 µg/mL ampicillin.
11. Into each 100-mm culture dish, add the content of one EB cell suspension tube.
12. Incubate for 16 h to give cells time to attach.
13. Replace the complete ES cell medium carefully with 10 mL (serum-free) high-glucose DMEM supplemented with N2.
14. Add into each culture dish 10 µL EGF stock solution and 10 µL IGF-1 stock solution for a final concentration of 20 ng/mL EGF and 50 ng/mL IGF-1.
15. Incubate for 10 d and replace medium on d 3, 6, and 9 with fresh high-glucose DMEM with N2, EGF, and IGF-1.
16. On d 10, add 20 µL bFGF stock solution.
17. Incubate until d 12 and continue with expanding the selected progenitors (*see* **Subheading 3.3.5.**).

3.3.5. Expansion of Inner Ear Progenitor Cells

1. Label five groups of five poly-L-ornithine 100-mm culture dishes, for example, five dishes with Pro1, five dishes with Pro2, and so on (*see* **Note 18**).
2. Put an additional gelatin coat on the poly-L-ornithine-coated culture dishes by adding 5 mL 0.1% gelatin into each dish and incubating at room temperature for 1–2 h.
3. Aspirate the medium from the five 100-mm dishes at d 12 of inner ear progenitor cell selection.
4. Add 1 mL 0.5X trypsin-EDTA to each dish. Tilt to cover the whole surface and incubate for 2–3 min at room temperature.
5. Check under the microscope to determine the moment when cells just start to detach from the surface.
6. Add 5 mL high-glucose DMEM medium (with 15% FBS and 100 µg/mL ampicillin) to each dish and very gently disrupt cell aggregates by pipetting with a 5-mL pipet. Do not completely dissociate the cells.
7. Transfer the cell suspensions into 15-mL conical tubes and collect the cells by centrifugation at 100*g* for 5 min.

8. Replace the gelatin solution in each of the 25 100-mm polyornithine-coated dishes with 9 mL high-glucose DMEM medium with 15% FBS and 100 µg/mL ampicillin.
9. When the centrifugation is complete, aspirate the supernatants without disturbing the cell pellets. Carefully resuspend the cells of each conical tube in 5 mL high-glucose DMEM medium with 15% FBS and 100 µg/mL ampicillin.
10. Distribute the resuspended cells from each tube evenly among each group of five identically labeled dishes.
11. Incubate for 10–14 h at 37°C in the cell culture incubator.
12. Carefully replace medium with high-glucose DMEM supplemented with N2, EGF, IGF-1, and bFGF (*see* **Note 19**).
13. Replace medium after 4 d.
14. At d 7, use one dish from each group for reverse transcriptase (RT) PCR gene expression analysis (*see* **Subheading 3.4.1.**).
15. Discard dishes of progenitor cell groups that do not display phenotypical characteristics of inner ear progenitor cells based on semiquantitative RT-PCR analysis.
16. At d 8, freeze the cells that grow in dishes belonging to groups that contain inner ear progenitor cells (four dishes for each of the five groups) in five aliquots per dish (*see* **Notes 11** and **20**).

3.3.6. Short-Term Maintenance of Inner Ear Progenitor Cells

Selected progenitors are thawed (*see* **Subheading 3.1.3.**) and maintained for up to five passages using the following protocol. The progenitor cell population is heterogeneous, and it is necessary to perform a semiquantitative gene expression analysis by RT-PCR from cells at each passage to monitor for the robust presence of key developmental inner ear genes (*see* **Subheading 3.4.1.**). The progenitors grow rapidly in the presence of bFGF. It is recommended to split the cells one or two times a week and to maintain them on 100-mm culture dishes:

1. At 1–2 h before passing the progenitor cells, take four 100-mm poly-L-ornithine-coated culture dishes (*see* **Note 18**), add 5 mL 0.1% gelatin into each dish, and let sit at room temperature to coat the surface of the dish with gelatin.
2. Aspirate medium from a 70–90% confluent progenitor cell population grown in a 100-mm dish.
3. Add 1 mL 0.5X trypsin-EDTA to each dish. Tilt to cover the whole surface and incubate for 3–5 min at room temperature.
4. Check under a microscope to determine the moment when cells just start to detach from the surface.
5. Add 5 mL high-glucose DMEM medium with 15% FBS and 100 µg/mL ampicillin and very gently dislodge cell aggregates by pipetting with a 5-mL plastic pipet. Do not completely dissociate the cells.
6. Transfer the cell suspension into a 15-mL conical tube and spin at 100*g* for 5 min.
7. During centrifugation, replace the gelatin solution in the four dishes with 10 mL high-glucose DMEM medium with 15% FBS and 100 µg/mL ampicillin.
8. When the centrifugation is complete, aspirate the supernatant without disturbing the cell pellet. Carefully resuspend the cells in 2 mL high-glucose DMEM medium with 15% FBS and 100 µg/mL ampicillin. Do not completely dissociate the cells.
9. Distribute 200 µL cell suspension each into two prepared 100-mm dishes and 500 µL cell suspension each into the remaining two dishes (*see* **Note 21**).
10. After 16–24 h, carefully replace the medium with high-glucose DMEM supplemented with N2, EGF, IGF-1, bFGF, and 100 µg/mL ampicillin.
11. Grow for 3–5 d, replace 70% of exhausted medium after 3 d.

12. After 5 d, use the cells from one dish for semiquantitative gene expression analysis by RT-PCR (*see* **Subheading 3.4.1.**).

3.3.7. In Vitro Differentiation to Generate Inner Ear Cell Types

In vitro differentiation of selected progenitor cells happens when growth factors are withdrawn from proliferating progenitor cell populations. This can be done in 100-mm culture dishes to generate cell material for RT-PCR analysis, or the cells can be differentiated in 35-mm cell culture dishes.

3.3.7.1. In Vitro Differentiation in 100-mm Dishes

1. Prepare 100-mm progenitor cell cultures of medium density as described in **Subheading 3.3.6.**, **steps 1–10**.
2. After 12–14 h growth in high-glucose DMEM supplemented with N2, EGF, IGF-1, and bFGF; replace the medium with high-glucose DMEM supplemented with N2 and 100 µg/mL ampicillin.
3. Culture for 14 d by replacing 50–70% of the exhausted medium every 2–3 d.
4. After 14 d, use the cells for semiquantitative gene expression analysis by RT-PCR (*see* **Subheading 3.4.1.**).

3.3.7.2. In Vitro Differentiation in 35-mm Four-Well Dishes

1. Prepare a cell suspension of selected inner ear progenitor cells as described in **Subheading 3.3.6.**, **steps 1–8** (*see* **Note 22**).
2. Remove a 100-µL aliquot of the cell suspension and add 900 µL high-glucose DMEM medium with 15% FBS and 100 µg/mL ampicillin. Split the cell suspension into two 500-µL aliquots.
3. Triturate one of the aliquots with a P-1000 pipet to completely dissociate the cells.
4. Determine the cell concentration of the dissociated cells with a hemocytometer.
5. Transfer the equivalent volume representing 10^5 cells from the nondissociated cell suspension into a 35-mm poly-L-ornithine-coated four-well dish filled with 1.5 mL high-glucose DMEM medium with 15% FBS and 100 µg/mL ampicillin (*see* **Note 23**).
6. After 16–24 h, replace the medium with high-glucose DMEM supplemented with N2 and 100 µg/mL ampicillin.
7. Culture for 14 d by replacing 50–70% of the exhausted medium every 2–3 d.
8. After 14 d, use the cells for immunocytochemistry (*see* **Subheading 3.4.2.**).

3.4. Screening for Phenotypic Markers

3.4.1. Comparative RT-PCR

3.4.1.1. Total RNA Preparation

Total RNA is isolated from cultured ES cells, selected progenitor cells, and differentiated cells using silica gel-based membrane spin columns (Qiagen RNeasy midikit). To ensure optimal RNA quality, it is recommended to isolate RNA from fresh cells grown on a 100-mm dish (*see* **Note 24**).

1. Carefully aspirate medium from ES, progenitor, or differentiated cells grown on a 100-mm dish and wash cells once with 10 mL PBS.
2. Add 1 mL 1X trypsin-EDTA to each dish. Tilt to cover the whole surface and incubate for 2–3 min at room temperature.

3. Check under a microscope to ensure that cells are coming loose and carefully tap on the side of the dish to aid detaching the cells.
4. Add 5 mL high-glucose DMEM medium with 15% FBS and 100 µg/mL ampicillin to the dish and gently disrupt cell aggregates by pipetting up and down with a 5-mL pipet.
5. Transfer the cell suspension into a 15-mL conical tube and spin at 200*g* for 5 min.
6. Aspirate the supernatant, very gently resuspend the cells in 12 mL PBS, and spin again at 300*g* for 5 min.
7. Completely aspirate the supernatant (*see* **Note 25**).
8. Loosen the pellet by flicking the tube and add 4 mL buffer RLT-β-ME; mix by pipetting up and down.
9. Vortex for 30 s and then homogenize by passing the lysate 15–20 times through a 21-gage needle fitted to a sterile, plastic 5-mL syringe.
10. Add 4 mL 70% ethanol to the homogenized lysates and mix by shaking vigorously.
11. Apply 4 mL sample to an RNeasy midicolumn placed in a 15-mL centrifuge tube and spin for 5 min at 4000*g*. Discard the flow through and apply the remaining 4-mL sample, spin again for 5 min at 4000*g*, and discard the flow-through.
12. Wash the column by applying 4 mL buffer RW1, spin for 5 min at 4000*g*, and discard the flow-through.
13. Add 2.5 mL buffer RPE to the column, spin for 3 min at 4000*g*, and discard the flow-through. Repeat this step once.
14. Transfer the column to a fresh 15-mL centrifuge tube and spin for 5 min at 4000*g* to dry the column.
15. Elute into a fresh 15-mL centrifuge tube by adding 250 µL RNase-free water directly onto the silica gel membrane of the column, let sit for 2 min, and then spin for 5 min at 4000*g*.
16. Combine 10 µL eluted RNA with 90 µL RNase-free water and measure the absorption at 260 nm (*see* **Note 26**).
17. Dispense RNA solution into 1.5-mL RNase-free microcentrifuge tubes in aliquots representing 10 µg nucleic acid; add 1/10 vol 3 *M* sodium acetate solution and 1.1 vol isopropanol. Mix by vortexing and store the RNA precipitate at –80°C.

3.4.1.2. Reverse Transcription

Screening for up- or downregulation of mRNAs by comparative RT-PCR works best when equal amounts of total RNA are used from the beginning. Good results have been achieved when starting with 10 µg-aliquots of ES, progenitor, or differentiated cell RNA stored at –80°C in the form of isopropanol precipitates (*see* **Note 27**). cDNA generated by reverse transcription can be used directly for PCR amplification.

1. Collect the precipitated RNA by centrifugation at 15,000*g* (or faster) at 4°C for 15 min.
2. Carefully remove and discard the supernatant and wash the pellet with 500 µL 70% ethanol.
3. Collect the RNA again by centrifugation at 15,000*g* (or faster) at 4°C for 15 min.
4. Carefully remove and discard the supernatant and dry the pellet for 5 min, or until dry, at low heat in a Speed Vac.
5. Add 12 µL oligo(dT) mix to the pellet and let sit on ice for 10 min; carefully flick the bottom of the tube every 2–3 min to help dissolve the pellet.
6. Heat at 65°C for 5 min and spin at 15,000*g* for 5 s to collect the liquid at the bottom of the tube.
7. Place the tube on ice and add 4 µL 5X first-strand buffer, 1 µL dNTP mix, 1 µL 0.1 *M* dithiothreitol solution; mix and spin shortly to collect the liquid at the bottom of the tube.

Table 1
Primer Pairs

Marker	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product length
Gapdh	AACGGGAAGCCCATCACC	CAGCCTTGGCAGCACCAG	442 bp
Otx2	CCATGACCTATACTCAGGCT TCAGG	GAAGCTCCATATCCCTGGG TGGAAAG	211 bp
Nestin	GCCGAGCTGGAGCGCGAG TTAGAG	GCAAGGGGGAAGAGAAG GATGTCG	694 bp
Pax2	CCAAAGTGGTGGACAAGA TTGCC	GGATAGGAAGGACGCTCA AAGAC	544 bp
BMP4	TGGTAACCGAATGCTGAT GGTCG	GTCCAGTAGTCGTGTGATG AGGTG	598 bp
BMP7	TGGGCTTCTGAGGAGGGC TGGTTG	TGGCGTGGTTGGTGGCGT TCAT	484 bp
Jagged-1	CAGAATGACGCCTCCTGTCG	TGCAGCTGTCAATCACTTCG	361 bp
$p27^{Kip1}$	CTGGAGCGGATGGACGCC AGAC	CGTCTGCTCCACAGTGCC AGC	525 bp
Math1	AGATCTACATCAACGCTCT GTC	ACTGGCCTCATCAGAGTC ACTG	449 bp
Myosin VIIA	CTCCCTCTACATCGCTCT GTTCG	AAGCACCTGCTCCTGCTCG TCCACG	628 bp
Espin	CAGCCTGAGTCACCGCA GCCTC	TGACCTGTCGCTGCCAGG GCGCG	475 bp
Brn3.1	GCCATGCGCCGAGTTTGTC	ATGGCGCCTAGATGATGC	368 bp
AchR α9	GAAGAACGTCATCTCCTAC GGCTG	CAGCTCTCACCCACATCG TAGAC	441 bp

8. Incubate at 42°C for 2–3 min and then add 1 µL (200 U) SuperScript II reverse transcriptase to the mixture.
9. Incubate at 42°C for 1 h.
10. Inactivate the enzyme by incubation at 70°C for 10 min.
11. Add 0.5 µL (1 U) RNase H to remove RNA (*see* **Note 28**).
12. Add 30 µL 10 m*M* Tris-HCl, pH 7.0; mix well and store the cDNA in 20 25-µL aliquots at –20°C until ready for PCR.

3.4.1.3. Polymerase Chain Reaction

Oligonucleotide primers for PCR should be selected to discriminate between cDNA and genomic DNA when possible. Using individual primers specific for different exons is a simple way to achieve this; amplification from genomic DNA with these primers will create a larger reaction product that often will not be efficiently amplified (*see* **Note 29**). **Table 1** lists several primers that have been successfully used to compare marker gene expression by ES cells, selected inner ear progenitor cells, and differentiated inner ear

cell types *(3,10)*. The following protocol is for one comparative (semiquantitative) PCR using three different samples, such as cDNA from ES cells, selected progenitors, and differentiated cells (*see* **Fig. 3**).

1. Thaw cDNA aliquots for the samples to be tested; keep the tubes on ice.
2. Prepare master mix for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (MM1) in a sterile 1.5-mL reaction tube on ice: 107.5 µL sterile MilliQ water; 17.5 µL 10X *Taq* reaction buffer; 17.5 µL DMSO; 7.0 µL 10 m*M* dNTP mix; 7.0 µL GAPDH forward primer (*see* **Note 30**); and 7.0 µL GAPDH reverse primer (*see* **Note 30**).
3. Mix by vortexing; spin shortly at 15,000*g* for 5 s to collect the liquid at the bottom of the tube and keep on ice.
4. Prepare master mix for a specific primer pair (MM2) in a sterile 1.5-mL reaction tube on ice: 107.5 µL sterile MilliQ water; 17.5 µL 10X *Taq* reaction buffer; 17.5 µL DMSO; 7.0 µL 10 m*M* dNTP mix; 7.0 µL specific forward primer (*see* **Note 30**); and 7.0 µL specific reverse primer (*see* **Note 30**).
5. Mix by vortexing; spin shortly at 15,000*g* for 5 s to collect the liquid at the bottom of the tube and keep on ice.
6. Distribute three 47.5-µL aliquots of MM1 and three 47.5-µL aliquots of MM2 into thin-wall PCR reaction tubes; keep the tubes on ice.
7. Add 2.5 µL of each specific cDNA (*see* **Subheading 3.4.1.2.**) to one MM1 aliquot and to one MM2 aliquot. For example, 2.5 µL ES cell cDNA are added to MM1, and 2.5 µL cDNA are added to MM2; the same is done for progenitor cell cDNA and for differentiated cell cDNA. Keep the tubes on ice.
8. Place the tubes directly into the preheated thermal cycler (idling at 94°C) and execute the following program (*see* **Note 31**): 94°C denaturation (60 s); *x* cycles of 94°C denaturation (30 s), 58°C annealing (30 s), and 72°C extension (60 s). The number of cycles *x* has to be pretermined in pilot experiments. With the instrumentation described here and the primers listed in **Table 1**, good results were obtained using 22 cycles for Otx2; 25 cycles for GAPDH; 30 cycles for myosin VIIA, espin, Brn3.1, and AchRa9; and 32 cycles for all other primer pairs (*see* **Note 32**).
9. Place samples on ice until all amplification reactions are completed.
10. Separate 15-µL aliquots of the reaction products on 2% agarose gels and compare intensity of the bands.

3.4.2. Immunocytochemistry

A few immunocytochemical markers can be used to identify the auditory precursors and the differentiating hair cells. The following is a general protocol that works well for all the antibodies mentioned (*see* **Table 2**). Special consideration should be given to the fixation because some epitopes will not tolerate aldehyde fixation. Such antibodies will require acetone-methanol fixation and have been identified in the text.

1. Aspirate the medium from in vitro-differentiated cells (after 14 d in culture as described in **Subheading 3.3.7.2.**) or proliferating progenitor cells (after 24 h in culture as described in **Subheading 3.3.7.2.**, **step 7**) grown in 35-mm four-well dishes. Wash the cells with PBS and fix the cells at room temperature with 4% paraformaldehyde solution for 15 min (*see* **Notes 33** and **34**).
2. Wash fixed cells once with PBS, permeabilize, and block with PBT1 for 15 min at room temperature (*see* **Note 35**).

Table 2
Selected Antibodies

Antibody to	Details	Supplier/reference
Nestin (mouse)	Mouse monoclonal, clone Rat-401 (recognizes mouse, does not crossreact with human)	Developmental Studies Hybridoma Bank, Iowa City, IA; cat. no. Rat-401
Nestin (human)	Mouse monoclonal, clone 10C2 (recognizes human, does not crossreact with mouse)	Chemicon, cat. no. MAB5326
Pax2	Rabbit polyclonal	Covance Research Products, Denver, PA; cat. no. PRB-276P
Myosin VIIa	Rabbit polyclonal	C. Petit, Institut Pasteur, Paris
Math1	Mouse monoclonal	Developmental Studies Hybridoma Bank, cat. no. Math-1
Espin	Rabbit polyclonal	A. J. Hudspeth, The Rockefeller University, New York ***(11)***

3. Incubate cells with the primary antibody in PBT1 for 2 h at room temperature or overnight at 4°C.
4. Wash the cells four times for 5 min each with PBT1.
5. Incubate cells with the fluorescence-conjugated secondary antibody in PBT2 for 1 h at room temperature. Shield from light.
6. Wash twice for 5 min each with PBT2.
7. Wash twice for 5 min each with PBS.
8. Mount with Slowfade or Vectashield mounting medium with DAPI (*see* **Note 36**), cover with 10-mm round cover slip, and suction away excess mounting medium with a pipet tip connected to a vacuum source with liquid trap.

4. Notes

1. All cell culture is done in a dedicated room, separated from the main laboratory by a closed door. Traffic in and out of the cell culture room has to be minimized. All supplies and instruments used for cell culture have to be dedicated and are never carried into the main laboratory and used for other experiments. To avoid contamination, all surfaces are wiped before and after use with 70% ethanol. Nothing inside the room is to be touched without wearing gloves. Sterile technique and common sense are usually effective means to avoid loss of cell lines because of contamination. It is recommended to use sterile plasticware instead of glassware.
2. ES cell-qualified FBS is used for maintenance of undifferentiated ES cells. It can be substituted with qualified FBS in all other experiments.
3. Heat inactivate serum in a 56°C water bath for 1 h; store frozen in 50- and 25-mL aliquots.
4. β-ME is toxic; dispense in a fume hood and wear protective clothing.
5. If a conventional water bath is used instead of a shaking water bath, then take out the tube every 1–3 min and thoroughly mix up the tissue pieces by flicking the tube approx 10 times with a finger.

6. When MEF feeders are prepared for hES cell maintenance, make sure that complete DMEM is used to seed the feeder layer. hES medium is not suitable for seeding MEF feeders.
7. Selected progenitor cells are kept only in complete medium to give the cells time to adhere. If cells are adherent already after 6–8 h, then carefully exchange the medium to serum-free conditions. Do not keep the cells in complete medium for an extended time.
8. For freezing ES cells, use ES cell-qualified cell culture freezing medium. For each batch of cells, dilute 2.5 mL 2X concentrate with 2.5 mL complete ES medium and keep on ice until use.
9. If ES or progenitor cells that are usually maintained without complete dissociation are cryopreserved, then the enzymatic digestion (with trypsin or collagenase) has to be carefully monitored to ensure for sufficient, but not complete, dissociation. Dissociation that results in approx 30% single cells and approx 70% clusters of 2–10 cells is optimal.
10. Some cell freezing protocols suggest that the cell concentration should be between 5×10^6 and 1×10^7 cells/mL. Resuspending MEFs, ES cells, or progenitor cells from a 75% confluent 100-mm culture dish in 5 mL cell culture freezing medium and freezing the cells in 0.5-, 0.75-, or 1-mL aliquots generally results in adequate recovery and sufficient viability of the cells after thawing.
11. Instead of using a Nalgene Cryo 1°C freezing container, which only has 18 slots for vials, one can assemble a makeshift freezing container for up to 100 vials by cooling 250–500 mL isopropanol to 4°C in a styrofoam box. Place the vials with the cells resuspended in freezing medium into small plastic bags. Use cryovials with color-coded lids because leaking isopropanol may erase any labeling on the tubes. Float the plastic bags on the isopropanol; cover the styrofoam box and place it into a –80°C freezer for 24 h.
12. It is recommended to keep approx 20–30% of the conditioned MEF medium.
13. In the beginning, it is recommended to seed a constant number of cells and to subsequently monitor cell density to develop a sense for the optimal dilution of cells that work best with the individual researcher's culture scheme. When counting ES cells recovered from a plate with inactivated MEFs, count only the small round ES cells that can easily be distinguished from the feeder cells.
14. It is important to monitor cell density and status of media exhaustion (pH indicator) and to intervene when the cells grow too dense or the media is exhausted too rapidly.
15. Alternatively, use a cell scraper (BD Falcon, BD Biosciences, cat. no. 353087) or five to eight sterile glass beads to lift colonies without creating a single-cell suspension.
16. The hES cells are generally split when they reach 70–80% confluency. Although split ratios vary, a 1:4 ratio is commonly used.
17. It helps to practice this technique first without cells and only a few drops.
18. Cell culture dishes are coated with poly-L-ornithine by covering the surface of each dish with poly-L-ornithine solution (use as little solution as necessary to cover the complete surface: approx 1 mL for 100-mm dishes, approx 250 µL for 35-mm dishes, or approx 80 µL per well of a four-well 35-mm dish). Coating happens overnight at 4°C. The following day, aspirate the poly-L-ornithine, wash the surfaces twice with excess sterile water, and let the dishes dry in the hood's sterile air current with open lids. Poly-L-ornithine-coated dishes are stored for up to 3 mo at 4°C.
19. Stock up on growth factors before expanding inner ear progenitors. This step requires a lot of growth factors.
20. Freezing all selected progenitor cells ensures that subsequent cultures are done with identical source material.

21. It is recommended always to plate selected inner ear progenitors in two densities and to monitor cell growth during subsequent days. Discard cultures that start with less than 30% confluency and cultures that grow too dense in just 2–3 d.
22. Prepare 35-mm dish cultures in parallel for routine maintenance of selected progenitor cells. The results obtained by RT-PCR of the progenitor cells are a good control to ensure that the source cells used for in vitro differentiation express the appropriate combination of developmental inner ear genes.
23. Each well of the 35-mm four-well dishes can be used for an individual immunostaining. It is recommended to prepare several four-well dishes to allow for single and double immunostainings and appropriate controls.
24. Because of the high stability and high efficacy of RNases, it is necessary to create an RNase-free environment by wiping the working area with RNaseZap. In addition, it is necessary to wear gloves while handling reagents and samples and to use RNase-free sterile, disposable plasticware at all experimental steps. For details on the following abbreviated protocol, refer to the RNeasy kit protocol booklet (provided with the kit).
25. It is important to completely remove the cell culture medium and to completely aspirate the PBS to avoid diluting the lysis buffer.
26. The RNA concentration in micrograms per milliliter can be calculated by multiplying the absorption with the dilution factor ***(10)*** and the constant 40. Expect a concentration between 150 µg and 1.0 mg/mL. If the measured absorption is greater than 2.5, then dilute more.
27. It is recommended to treat all samples that are going to be compared equally and in one batch of experiments conducted together.
28. Removal of RNA, particularly of RNA bound to cDNA (RNA:DNA hybrids), will increase the sensitivity of subsequent PCR reactions.
29. It is important to establish specific PCR conditions (cycling parameters) for each of the different products that are optimized to generate products at the linear portion of the product accumulation curve. These parameters depend in part on the thermal cycler used in the experiment, and it is recommended to conduct a series of pilot experiments for each primer pair with all samples that will be compared (e.g., cDNA from ES cells, selected progenitors, and differentiated cells). Cycling parameters need to be selected based on the sample that produces the highest amount of amplification product.
30. Custom-synthesized oligonucleotide primers are usually shipped lyophilized. Resuspend primers in sterile MilliQ water at a concentration of 100 p*M*/µL.
31. It is convenient to program the thermal cycler to cool the reactions at 4°C once the amplification is done. Because of the different cycle numbers of the different reactions, close monitoring of the reaction is required to ensure that samples are removed from the thermal cycler at the appropriate time points.
32. The thermal cycler has to be programmed to run the program with the most cycles. Reactions that undergo fewer cycles have to be removed at appropriate time points at the end of the 72°C extension period.
33. For aldehyde-sensitive epitopes, fix for 2 min with a cold (–20°C) 50:50 acetone-methanol mixture. Let air-dry for 5 min after fixation.
34. It is preferable to label the cells directly on plastic as this is the condition they are normally grown, and the results are, in principle, more comparable to the ones obtained by other techniques such as RT-PCR or cell sorting. Use of four-well 35-mm dishes has the advantage that cells can be stained in a small volume (100 µL), which conserves reagents. Alternatively, regular 35-mm cell culture dishes can be used. The borders of the plastic

dishes can be trimmed with a hot knife or broken off with a pair of pliers to allow access of the higher magnification objectives to the edges of the field.

35. Acetone-methanol-fixed cells do not need to be permeabilized because the membranes have been dissolved. However, the inclusion of Triton X-100 in the incubation and washing steps helps to reduce background.
36. Alternatively, mount with 50% glycerol in PBS with or without 10 µg/mL DAPI.

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6

Derivation of Epidermal Colony-Forming Progenitors From Embryonic Stem Cell Cultures

Tammy-Claire Troy and Kursad Turksen

Summary

In our two-step culturing system, epidermal progenitor cells derived from differentiating embryonic stem cells in vitro are plated on either a basement membrane matrix or in co-culture. On plating on basement membrane, differentiation along the epidermal lineage is evident and reproducible, with several markers of epidermal differentiation observed in a pattern reminiscent of their in vivo expression. However, when epidermal progenitor cells are plated in a co-culture environment with mature epidermal cells at low density, colony formation, and differentiation along the epidermal pathway is enhanced with a high frequency of K14-positive cells. Thus, the in vitro co-culture model system described here is useful in dissecting the process of commitment and differentiation of epithelial progenitors that occurs during epidermogenesis as well as for the investigation of the molecular mechanisms underlying these processes.

Key Words: Co-culture; embryonic stem (ES) cells; epidermal progenitor cells (EPCs); epidermogenesis; keratin 14 (K14); keratinocytes.

1. Introduction

Embryonic stem (ES) cells derived from 3.5-d-old mouse blastocysts can be maintained indefinitely in an undifferentiated, pluripotent state when cultured under strict conditions *(1–3)*. With the introduction of specific culturing conditions and growth factors, ES cells have been shown to differentiate faithfully into myriad cell lineages, including epidermal cells, contracting muscle cells, neurons, adipocytes, and chondrocytes (to name only a few); for an extensive list of lineages that have been developed, *see* **ref.** *4* and this volume. An in vitro ES cell culture system that would allow us to study the skin and its appendages (sebaceous glands and hair follicles) would be invaluable for understanding the mechanisms involved in the early steps of commitment and differentiation of epidermal stem cells. We have designed a modified culturing protocol in which this differentiation can be achieved through a two-step process, an advancement over our previously described method *(3,5,6)*.

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Epidermal progenitor cells (EPCs) are derived from secondary cultures of differentiating embryoid bodies (dEBs) that are produced from pluripotent ES cells. EPCs undergo commitment and differentiation to give rise to keratin 14 (K14)-positive basal-like epidermal cells in vitro. We have found that EPCs require specific culture conditions to optimize this commitment and differentiation toward the epidermal lineage *(7)*; however, it became apparent that the efficiency of these cultures remains to be improved.

There are specific factors, which we describe here, that play an important role in the generation of K14-positive cells: cell density and culturing conditions. Various cell densities were plated on 35-mm tissue culture dishes and were cultured on either basement membrane (BM) matrix or in co-culture with mature keratinocytes. The epithelial nature of the differentiating EPCs was confirmed by Ayoub-Shklar (AS) staining and immunohistochemical staining for K14. It is evident that cell density and culturing conditions are essential factors that influence the differentiation of EPCs along the epidermal pathway, and that cell survival and the ability to produce K14 are tightly regulated by these factors.

Our observations lead us to the conclusion that EPCs are highly dependent on the community effect for their survival and differentiation into the epidermal lineage. Together with the unidentified signals and growth factors produced by mature keratinocytes, the survival, proliferation, and differentiation of EPCs are therefore enhanced by our two-step co-culturing protocol.

2. Materials

2.1. Tissue Culture

1. Cell counter (Beckman Coulter, Mississauga, Canada; model no. Z2).
2. 22 × 22 mm cover slips (Bellco, Vineland, NJ; cat. no. 1916-12222).
3. 500 mL Dulbecco's modified Eagle's medium (DMEM) (1X; Invitrogen, Burlington, Canada; cat. no. 11960-044).
4. 500 mL fetal bovine serum (FBS) characterized and screened for ES cell growth (Hyclone, Logan UT; cat. no. SH30071.03) (*see* **Note 1**).
5. Glass pipets (10 and 25 mL).
6. Humidified incubator at 37°C and 5% CO_2.
7. 10 mL insulin-transferrin-selenium (ITS) (100X; Invitrogen, cat. no. 41400-045).
8. Inverted microscope with phase contrast objectives (×10 to ×25) equipped with photographic capabilities.
9. 500 mL keratinocyte growth medium (KGM) without Ca^{2+} (Cambrex, East Rutherford, NJ; cat. no. CC-3112).
10. Laminar flow cabinet.
11. 10 mL Matrigel (growth factor-reduced BM matrix) (VWR, Mississauga, Canada; cat. no. CACB35-4230) (*see* **Note 2**).
12. 2 mg mitomycin C (Roche Diagnostics, Mississauga, Canada; cat. no. 107409) (*see* **Note 3**): to prepare, add 5 mL 1X phosphate-buffered saline (PBS) to a 2-mg vial, recap, and shake vigorously. Empty the vial into a weigh boat and syringe filter. Prepare 500-µL aliquots and store at –20°C for up to 6 mo.
13. 100 mL 10 m*M* nonessential amino acids (NEAA) (100X; Invitrogen, cat. no. 11140-050).

14. 1X PBS: prepare from 10X PBS, then aliquot into 100-mL bottles and autoclave. To prepare 10X PBS (1 L), dissolve 11.5 g sodium phosphate dibasic (Na_2HPO_4) and 2.0 g potassium phosphate monobasic (KH_2PO_4) in 500 mL distilled water (dH_2O). Add 80 g sodium chloride (NaCl) and 2 g potassium chloride (KCl) and adjust volume to 1 L with distilled water.
15. 100 mL penicillin-streptomycin (100X; Invitrogen, cat. no. 15140-122).
16. 100-mm Petri dishes (Fisher Scientific, Nepean, Canada; cat. no. 08-757-12).
17. Pipetmen (2, 10, 20, 100, 200, and 1000 µL).
18. 15-mL polypropylene conical tubes (Beckton Dickenson, Oakville, Canada; cat. no. 2097).
19. 100 mL 100 m*M* sodium pyruvate (100X; Invitrogen, cat. no. 11360-070).
20. 0.2-µm syringe filters (VWR, cat. no. 28196-089).
21. 5-cc syringes (Becton Dickenson, cat. no. 309603).
22. Tabletop centrifuge.
23. Polystyrene tissue culture dishes (35, 60, and 100 mm; Corning, Corning, NY; cat. no. 25000-35, 25010-60, and 25020-100, respectively).
24. 500 mL 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (1X; Invitrogen, cat. no. 25200-072).
25. 20 mL type I collagen solution from calf skin (Sigma-Aldrich, Oakville, Canada; cat. no. C-8919).
26. 37°C water bath.

2.1.1. Media

1. 15% DMEM: DMEM is supplemented with 15% heat-inactivated FBS, 1% sodium pyruvate, 1% NEAA, and 1% penicillin-streptomycin. To a 500-mL bottle of DMEM, add 90 mL heat-inactivated FBS, 6 mL penicillin-streptomycin, 6 mL NEAA, and 6 mL sodium pyruvate.
2. KI2: KGM is supplemented with 1% ITS and 2% heat-inactivated FBS. For 100 ml, combine 97 mL KGM, 1 mL ITS, and 2 mL heat-inactivated FBS.

2.1.2. Cells

1. R1 ES cells *(8)* (kindly provided by Dr. Janet Rossant and Dr. Andras Nagy, University of Toronto, Toronto, Canada).
2. $K14^{-/-}$ keratinocytes *(9)*.

2.2. AS Staining

1. Acid fuchsin (Sigma-Aldrich, cat. no. A 3908).
2. 5% acid fuchsin: for 100 mL, add 5 g acid fuchsin to 100 mL dH_2O and stir overnight. Filter and store at room temperature for up to 6 mo. Filter before use.
3. Aniline blue (Polysciences Inc., Warrington, PA; cat. no. 02570).
4. Aniline blue-orange G: for 100 mL, mix 0.5 g aniline blue, 2 g orange G, and 1 g phosphotungstic acid in 100 mL dH_2O. Stir overnight, filter, and store at room temperature for up to 6 mo. Filter before use.
5. 95% ethanol.
6. Filter paper and funnels.
7. Formaldehyde solution.
8. 37% formalin: for 100 mL, combine 90 mL 1X PBS and 10 mL 37% formaldehyde.
9. Orange G (Sigma-Aldrich, cat. no. O 7252).
10. 1X PBS (*see* **Subheading 2.1.**, **item 14**).
11. Phosphotungstic acid (Sigma-Aldrich, cat. no. P-6395).

2.3. Immunofluorescence

1. 25 g 1,4-diazabicyclo[2. 2. 2]octane (DABCO) (Sigma-Aldrich, cat. no. D-2522).
2. Goat antirabbit immunoglobulin G, $F(ab')_2$ fragment specific, conjugated to fluorescein isothiocyanate (Cedarlane, Hornby, Canada; cat. no. 111-096-047).
3. Humidified chamber (*see* **Note 4**).
4. K14 antibodies (gift of Dr. Elaine Fuchs, Rockefeller University, New York, NY).
5. Methanol (stored at –20°C).
6. 500 g Mowiol 4-88 (Polysciences Inc., cat. no. 17951): for 20 mL, combine 2.4 g Mowiol 4-88 with 6 g glycerol and stir for 2 h at room temperature. Add 6 mL dH_2O and stir for 2 h at room temperature. Add 12 mL 0.2 *M* Tris-HCl at pH 8.5 and heat to 50°C for 10 min with occasional mixing. Using a disposable pipet, transfer the solution to a centrifuge tube and spin at 5000*g* for 15 min. Add 2.5% DABCO to reduce fading. Stir to dissolve. Aliquot into 1-mL tubes and store at –20°C. Thaw a tube as needed; may be stored for 2 wk at 4°C.
7. 1X PBS (*see* **Subheading 2.1.**, **item 14**).
8. 0.2 *M* Tris-HCl at pH 8.5.

3. Methods

Detailed protocols for the freezing and thawing of cells as well as the isolation and maintenance of embryonic fibroblasts (EFs) was previously detailed ***(6)***.

3.1. ES Cells

3.1.1. Feeder Layer Preparation

1. Add 250 µL mitomycin C to a 100-mm confluent EF dish containing 10 mL medium and agitate back and forth and side to side.
2. Incubate at 37°C and 5% CO_2 for 2–3 h.
3. Rinse the dishes three times with 10 mL 1X PBS, then replace the medium with 10 mL 15% DMEM.
4. Place the dishes in the incubator until the trypsinized cell suspension is prepared (*see* **Subheading 3.1.2.**, **step 5**).

3.1.2. ES Cell Culture

1. Gently remove the medium from a subconfluent 100-mm dish of ES cells growing on a mitotically inactivated EF feeder layer and rinse with 10 mL 1X PBS.
2. Add 2 mL 0.25% trypsin-EDTA and agitate the dish back and forth and side to side.
3. Return the plate to the incubator for 1–2 min until the cells float with gentle agitation. While waiting for trypsinization to be complete, add 10 mL 15% DMEM to 100-mm Petri dishes (*see* **Note 5**) for the generation of embryoid bodies (EBs).
4. When the ES cells are floating, gently pipet up and down 30 times with a 1-mL Pipetman to make a single-cell suspension.
5. Distribute 200 µL (approx 2.5×10^6 cells) to each prepared feeder layer (from **Subheading 3.1.1.**) and Petri dish, making a 1:10 dilution of the original culture.
6. Gently agitate up and down and side to side and incubate at 37°C and 5% CO_2.
7. Change the medium of the ES cells plated on the EF feeder layer every 2 d and subculture every 3–4 d (*see* **Note 6**). ES cells plated in suspension culture in Petri dishes quickly form EB cell aggregates (*see* **Subheading 3.2.**).

3.2. EB Culture

1. Change the medium of EBs in suspension (*see* **Subheading 3.1.2.**, **step 5**) on the third day of culture by collecting the entire contents of the Petri dish into a 15-mL polypropylene conical tube with a 10-mL pipet.
2. Allow EBs to settle by gravity and suction off the medium.
3. Gently resuspend the cells in 10 mL 15% DMEM.
4. Transfer the cells to the original Petri dish and return the dish to the incubator.

3.3. Differentiating EBs

After 6 d in culture, EBs are plated on BM-coated plates for cell migration and the formation of epithelial sheets.

3.3.1. BM-Coated Plates

1. Coat 60-mm tissue culture dishes with 2 mL BM for 30 min at room temperature.
2. After 30 min, suction off the BM and gently replace it with 4 mL 15% DMEM (*see* **Note 7**).
3. Place the dishes into the incubator until the EBs are collected (*see* **Subheading 3.3.2.**, **step 2**).

3.3.2. dEB Cultures

1. After 6 d of EB formation, collect EBs as described in **Subheading 3.2.**, **steps 1–3**.
2. Gently distribute 1 mL EB suspension to BM-coated 60-mm dishes (*see* **Subheading 3.3.1.**) (mixing frequently) to make a 1:10 dilution of the suspension culture (*see* **Note 8**).
3. Return the cells to the incubator and allow them to adhere and spread for 4 d.
4. Change the medium on the second day.

3.4. Epithelial Progenitor Cells

1. After 4 d in culture, trypsinize dEBs (*see* **Subheading 3.3.2.**) with 2 mL 0.25% trypsin-EDTA for 2–3 min in the incubator.
2. Collect the cells in 15% DMEM into a 15-mL conical tube.
3. Remove 100 µL for counting.
4. Centrifuge at 700*g* for 2–3 min to pellet the cells. While centrifuging, count the cells using a cell counter.
5. Resuspend the pellet with 15% DMEM and dilute the cells appropriately to plate the desired number of cells (*see* **Subheading 3.5.**).
6. Plate EPC cells in co-culture (*see* **Subheading 3.5.1.**) or on BM substrata (*see* **Subheading 3.5.2.**) to induce differentiation.

3.5. Differentiation of EPCs Plating Conditions

EPCs were cultured at various cell densities (10^6, 0.5×10^6, 2.5×10^5, 10^5, 5×10^4, and 10^4 cells/35-mm dish) to determine if cell density played an important role in their differentiation. In addition, cells were plated under two different conditions that promote their differentiation toward the epidermal lineage: BM matrix and co-cultured with mature keratinocytes.

3.5.1. Mouse Keratinocyte Co-Culture

1. On the day of EPC culture, add 50 µL mitomycin C solution to the 2 mL medium in the co-culture plates (*see* **Subheading 3.6.4.**) and agitate back and forth and side to side.
2. Incubate at 37°C and 5% CO_2 for 2–3 h.

3. Rinse the dishes three times with 2 mL 1X PBS, then replace the medium with 1 mL 15% DMEM.
4. Return the plates to the incubator until the trypsinized cell suspension is prepared (*see* **Subheading 3.4.**, **step 6**).

3.5.2. BM Substrata

1. On the day of EPC culture, place 22 × 22-mm cover slips into the bottom of 35-mm tissue culture dishes (*see* **Note 9**).
2. Coat with 2 mL BM for 30 min at room temperature.
3. After 30 min, suction off the BM and replace medium with 1 mL 15% DMEM (*see* **Note 7**).
4. Put the coated dishes in the incubator until the cell suspension is available (*see* **Subheading 3.4.**, **step 6**).

3.6. Mouse Keratinocyte Cells

Because we are primarily interested in the expression of K14 as a marker for epithelial differentiation, the co-culture system we employ utilizes a mitotically inactivated $K14^{-/-}$ mouse keratinocyte cell (MKC) line isolated from the back skin of K14 knockout mice *(9)*. Our initial studies suggested that the mature keratinocytes provide factors that enhance the differentiation of EPCs along the epithelial cell fate.

3.6.1. Collagen Type I-Coated Plates

1. Coat 60-mm tissue culture plates with 100 µL collagen type I solution in 2 mL 1X PBS overnight in the 37°C, 5% CO_2, incubator.
2. Carefully rise the dishes three times with 1X PBS.
3. Add 4 mL KI2 medium to each dish. Return the plates to the incubator until the cell suspension is ready (*see* **Subheading 3.6.2.**, **step 4**).

3.6.2. MKC Culture

1. Trypsinize a 60-mm dish of confluent MKCs with 2 mL 0.25% trypsin-EDTA for 2–3 min in the 37°C, 5% CO_2, incubator until the cells detach with gentle agitation.
2. Collect the cells with 3 mL KI2 medium into a 15-mL conical tube and centrifuge at 700*g* for 2–3 min for pellet formation.
3. Carefully suction off the medium and resuspend the pellet in 5 mL KI2.
4. Distribute 1 mL (approx 2×10^5 cells) to each of the pre-prepared collagen-coated plates (*see* **Subheading 3.6.1.**), making a 1:5 dilution of the original culture.
5. Change the medium every other day.
6. Subculture after 3–4 d.

3.6.3. Plate Preparation for Co-Culture

1. At 2 d prior to EPC culture, place 22 × 22-mm cover slips into 35-mm tissue culture dishes (*see* **Note 9**).
2. Coat with 50 µL collagen type I solution in 1 mL 1X PBS overnight in the 37°C, 5% CO_2, incubator.
3. At 1 d prior to EPC culture, carefully rinse the dishes three times with 1X PBS.
4. After rinsing, put 1 mL KI2 medium in the plate and return plate to incubator until trypsinized cell suspension is prepared (*see* **Subheading 3.6.4.**, **step 4**).

3.6.4. MKCs for Co-Culture

1. Trypsinize MKCs with 2 mL 0.25% trypsin-EDTA for 2–3 min in the 37°C, 5% CO_2, incubator until the cells detach with gentle agitation.
2. Collect the cells with 3 mL KI2 medium into a 15-mL conical tube and remove 100 µL for counting.
3. Centrifuge at 700*g* for 2–3 min to pellet the cells. While centrifuging, count the cells using a cell counter.
4. Resuspend the cell pellet at 10^5 cells/mL and plate 1 mL onto the prepared dishes.
5. Incubate overnight before use in co-culture (*see* **Subheading 3.5.1.**).

3.7. Analysis of Differentiation

3.7.1. Phase Contrast Photography

It is evident that cell density plays a very important role in the survival and proliferation of EPCs independent of the culture conditions used. In each case, the greater the initial cell density used, the more cells survive and proliferate to produce EPC colonies. It is also apparent that EPCs plated on BM at too low a density have poor plating efficiency and, therefore, are not capable of producing many colonies. This affect is compounded by the slow proliferative ability of these cells because of the lack of cell–cell communication. In comparison, when the same low density is plated using an MKC co-culture system, healthy colonies are generated, and cells proliferate at a greater rate (*see* **Fig. 1**).

1. Fix cells for 10 min with 10% formalin at room temperature.
2. Rinse with 1X PBS and leave a fresh aliquot of 1X PBS.
3. Observe and photograph.

3.7.2. AS Staining

AS stain is very useful for determining the extent of differentiation of epidermal cells (***3,10***). Undifferentiated epidermal cells are blue; differentiated epidermal cells are red, and further differentiation is indicated with a color change from orange to yellow to brown. It is evident that cell density is important for the differentiation capabilities of EPCs. In each case, the greater the initial cell density used, the more they are able to differentiate. It is also apparent that EPCs plated on BM at too low a density have poor differentiation capacity, as indicated by the first appearance of differentiated colonies only after 6 d in culture. In comparison, when the same low density is plated using an MKC co-culture system, differentiated colonies are generated by d 4. By d 6, even some of the lower density colonies have differentiated (*see* **Fig. 2**).

1. Set up EPC cultures either on BM or in MKC co-culture conditions without cover slips.
2. At various time points, fix cells for 30 min with 10% formalin.
3. Apply 5% acid fuschin for 3 min.
4. Remove excess stain and incubate cells in aniline blue-orange G for 45 min.
5. Rinse cells with 95% ethanol and photograph with an inverted microscope equipped with a ×10 objective and phase contrast.

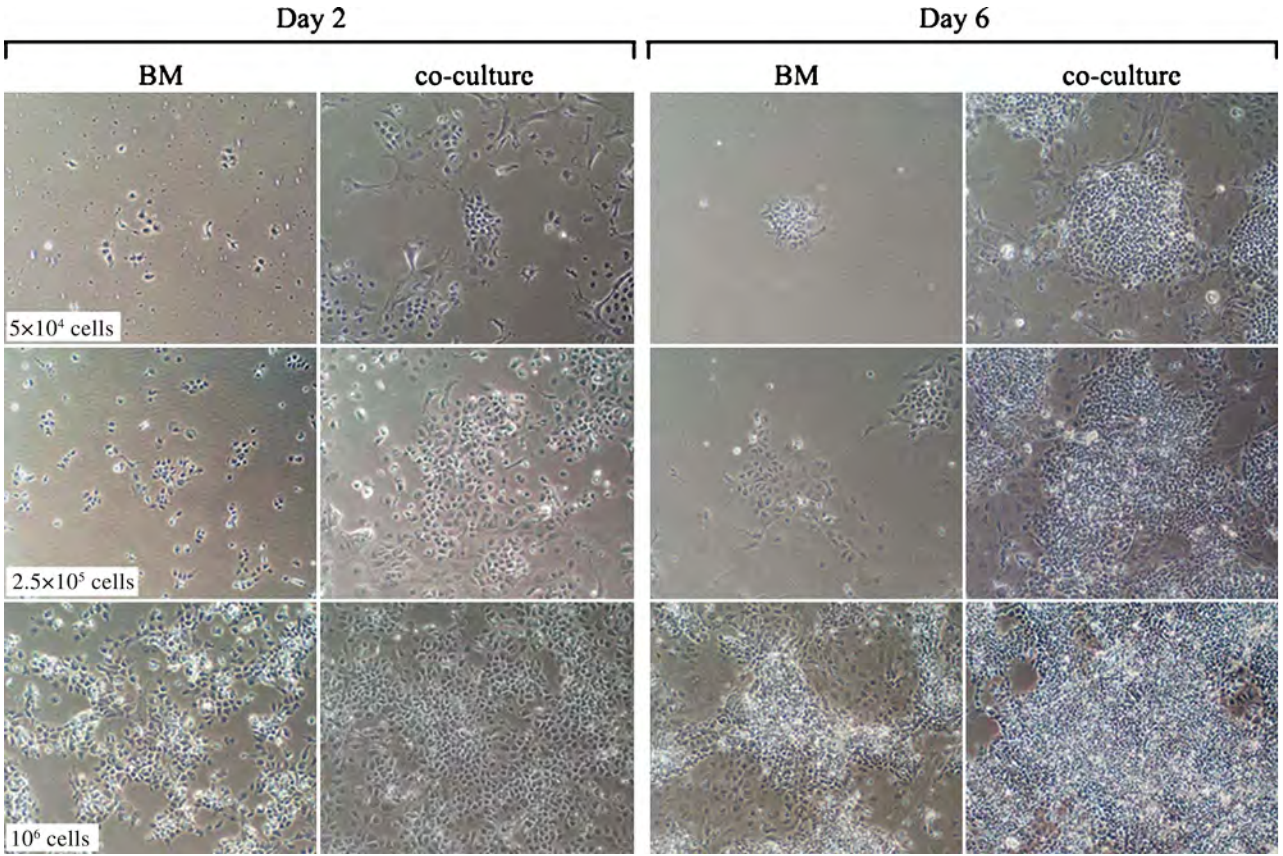

Fig. 1. Epidermal progenitor cells at various cell densities (5×10^4, 2.5×10^5 and 10^6 cells) plated on basement membrane as compared to using an mouse keratinocyte cell co-culture system after 2 and 6 d in culture. Epithelialization is increased with density and time in culture and is enhanced when the co-culture system is used. (Please *see* the companion CD for the color versions of this figure.)

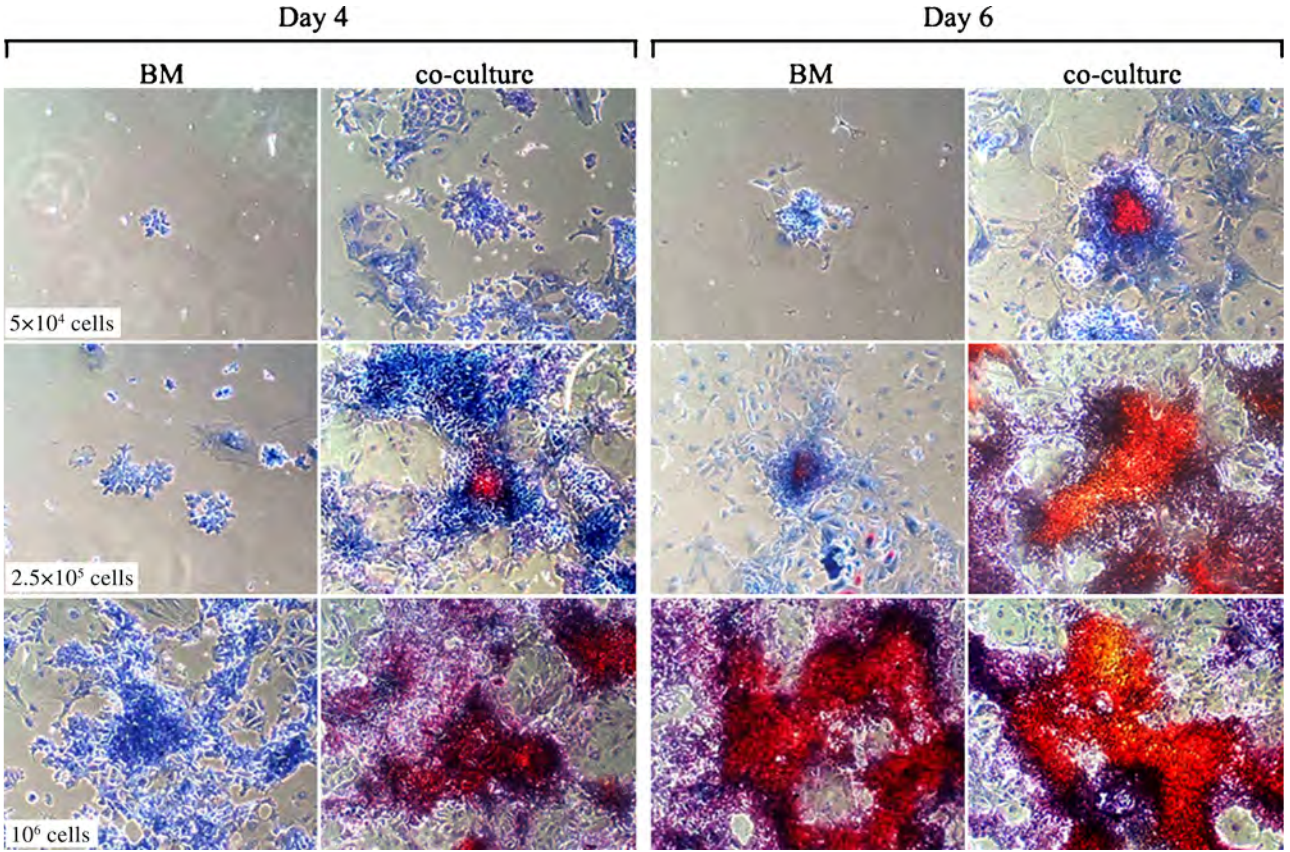

Fig. 2. Ayoub-Shklar staining of various cell densities of epidermal progenitor cells (5×10^4, 2.5×10^5 and 10^6 cells) plated on basement membrane (BM) as compared to using an mouse keratinocyte cell co-culture system after 4 and 6 d in culture. Differentiated epithelial cells were observed in the co-culture system at mid- to high densities after only 4 d in culture and even at very low density after 6 d in culture, much enhanced over the BM conditions. (Please *see* the companion CD for the color versions of this figure.)

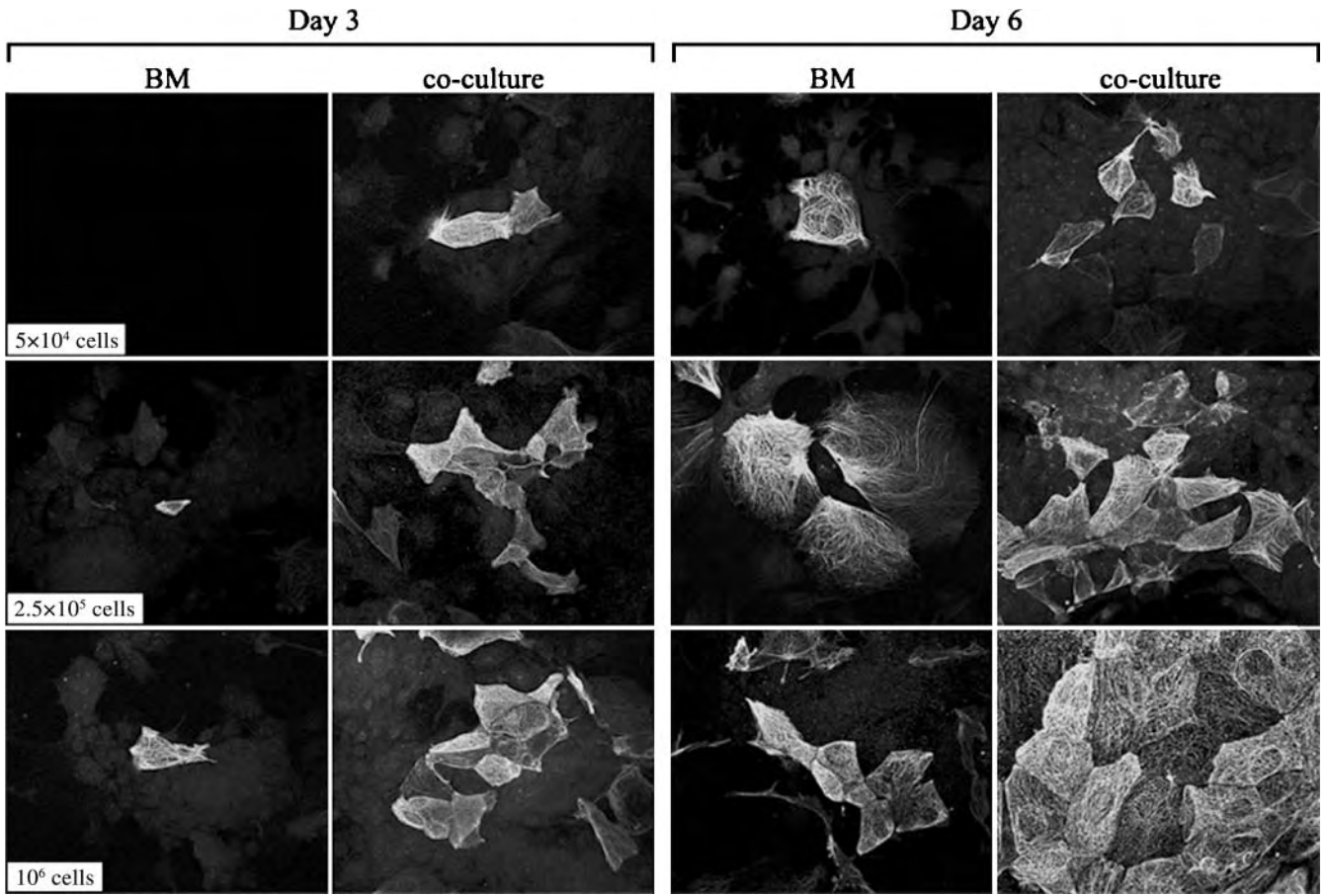
Day 3
Day 6
BM
co-culture
BM
co-culture
5×10⁴ cells
2.5×10⁵ cells
10⁶ cells

Fig. 3.

3.7.3. Immunohistochemistry

When plated on BM, a few K14-positive cells are evident after 3 d in culture, and small clusters of positive cells exist after 6 d in culture. In the co-culture system, however, after 3 d there are a number of K14-positive cells forming clusters at various densities. After 6 d in culture, epithelial sheets of K14-positive cells are observed at mid- to high densities; at low density, there exist clusters of K14-positive cells (*see* **Fig. 3**).

1. Set up EPC cultures either on BM or in MKC co-culture conditions on cover slips.
2. After different culturing periods, fix cells with ice-cold methanol for 10 min at –20°C.
3. Rinse the cover slips in three changes of 1X PBS.
4. Apply 100 µL K14 antibodies (diluted 1:100 in 1X PBS) to the cover slip and incubate for 30 min in a humidified chamber at room temperature.
5. Rinse three times in 1X PBS to remove unbound primary antibody.
6. Apply 100 µL antirabbit secondary antibodies (diluted 1:50 in 1X PBS) and incubate for 30 min in a humidified chamber at room temperature.
7. Rinse three times in 1X PBS.
8. Apply Mowiol 4-88 (approx 30 µL) on each mounting slide and carefully invert the cover slip on the slide.
9. Observe and photograph.

4. Notes

1. Heat inactivate serum in a 57°C water bath for 30 min, mixing every 10 min. Under sterile conditions, aliquot into 100-mL bottles and store at –20°C for 6–12 mo.
2. Thaw BM in a beaker of distilled water and aliquot 1 mL into sterile tubes with a chilled pipet; store at –20°C until required. To use, thaw a tube, add 9 mL unsupplemented DMEM, and further dilute BM 1:10 to a final working concentration of 0.1 mg/mL. Store at 4°C.
3. To thaw for use, wrap the tube in foil to protect the mitomycin C from light and warm in a 37°C water bath. Be aware that mitomycin C is toxic.
4. To prepare a humidified chamber for immunofluorescence, place a wet piece of filter paper in a 15-cm glass dish with a lid.
5. We have found that Fisher brand Petri dishes are best for the generation of EBs as they prevent EB sticking and aggregation.
6. It is important not to keep ES cells in culture for long periods to maintain pluripotency. Extensive culturing will result in abnormal karyotypes and inconsistent differentiation. Therefore, we generally maintain ES cells in culture only to the sixth passage.
7. Be careful not to disrupt the integrity of the matrix layer.
8. It is important to mix the EBs frequently while plating as they tend to sink quickly in the tube because of their mass.
9. If cover slips are not required, such as for AS staining, then simply coat the 35-mm dishes.

Fig. 3. Immunohistochemical analysis of keratin 14 (K14) expression of epidermal progenitor cells of various cell densities (5×10^4, 2.5×10^5 and 10^6 cells) plated on basement membrane (BM) as compared to using an mouse keratinocyte cell co-culture system after 3 and 6 d in culture. Single cells with K14 expression are evident after 3 d in BM cultures with K14-positive cell clusters emerging after 6 d. In co-culture, however, K14-positive cell clusters are evident after only 3 d in culture, and after 6 d, epithelial sheets of K14-positive cells are observed.

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7

Directing Epidermal Fate Selection by a Novel Co-Culture System

Tammy-Claire Troy and Kursad Turksen

Summary

We describe an improved culturing protocol to direct embryonic stem (ES) cell differentiation along the epidermal lineage in vitro. Pluripotent ES cells co-cultured with mitomycin C-treated mature epidermal cells at low density resulted in colonies that undergo differentiation to keratin 14 (K14)-positive cells within 3 d in culture. This new protocol suggests that the commitment and differentiation of undifferentiated ES cells can be efficiently directed by mature epidermal cell-derived factors. In addition, it suggests that this condition may be responsible for the maintenance and expansion of putative epidermal stem cells in vitro. Our advanced culturing regimen allows for the analysis of colony formation from a single cell to determine the factors controlling the differentiation capacities of very early progenitor cells in culture.

Key Words: Co-culture; embryonic stem (ES) cells; keratin 14 (K14); keratinocytes.

1. Introduction

Embryonic epidermal stem cells derived from differentiating embryonic stem (ES) cells undergo commitment and differentiation under certain conditions, giving rise to keratin 14 (K14)-positive basal-like epidermal cells in vitro. Our culture conditions that promote commitment and differentiation in a reproducible fashion recapitulate the expression of epidermal markers in vitro *(1–3)*. However, our original two-step protocol has been observed to be time consuming.

Therefore, improvements in the protocol have been developed recently to address this issue and to further determine the factors that play a role in the generation and maintenance of putative epidermal stem cells as well as their commitment and differentiation in vitro. Via the two-step process described in Chapter 6 of this volume, single-cell populations containing epidermal stem cells prepared from differentiating embryoid bodies were derived, and epidermal progenitor cells form colonies that differentiate to produce K14 when plated on basement membrane matrix. When plated in

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co-culture with committed mouse keratinocyte cells (MKCs), the differentiation potential is enhanced.

Therefore, we explored the development of a one-step culturing process by which pluripotent ES cells are plated directly in an MKC co-culture environment. The resulting cultures are capable of colony formation and the expression of K14 within 3 d in culture. This unique culturing protocol provides evidence that ES cell differentiation along the epidermal lineage requires signals from neighboring cells provided from our culturing regimen. Furthermore, it provides for the analysis of colony-forming units from a single ES cell to trace the signals controlling the differentiation process along the epidermal lineage in vitro.

2. Materials

2.1. Tissue Culture

1. Cell counter (Beckman Coulter, Mississauga, Canada; model no. Z2).
2. 22 × 22-mm cover slips (Bellco, Vineland, NJ; cat. no. 1916-12222).
3. 500 mL Dulbecco's modified Eagle's medium (DMEM) (1X; Invitrogen, Burlington, Canada; cat. no. 11960-044).
4. 500 mL fetal bovine serum (FBS) characterized and screened for ES cell growth (Hyclone, Logan UT; cat. no. SH30071.03) (*see* **Note 1**).
5. Glass pipets (10 and 25 mL).
6. Humidified incubator at 37°C and 5% CO_2.
7. 10 mL 100X insulin-transferrin-selenium (ITS) (Invitrogen, cat. no. 41400-045).
8. Inverted microscope with phase contrast objectives (×10 to ×25) equipped with photographic capabilities.
9. 500 mL keratinocyte growth medium (KGM) without Ca^{2+} (Cambrex, East Rutherford, NJ; cat. no. CC-3112).
10. Laminar flow cabinet.
11. 2 mg mitomycin C (Roche Diagnostics, Mississauga, Canada; cat. no. 107409): to a 2-mg vial, add 5 mL 1X PBS, recap, and shake vigorously. Empty the vial into a weigh boat and syringe filter. Prepare 500-µL aliquots and store at –20°C for up to 6 mo (*see* **Note 2**).
12. 100 mL 10 m*M* nonessential amino acids (NEAA) (100X; Invitrogen, cat. no. 11140-050).
13. 1X PBS: prepare from 10X PBS, then aliquot into 100-mL bottles and autoclave. To prepare 10X PBS (1 L), dissolve 11.5 g sodium phosphate dibasic (Na_2HPO_4) and 2.0 g potassium phosphate monobasic (KH_2PO_4) in 500 mL distilled water (dH_2O). Add 80 g sodium chloride (NaCl) and 2 g potassium chloride (KCl) and adjust volume to 1 L with distilled water.
14. 100 mL penicillin-streptomycin (100X; Invitrogen, cat. no. 15140-122).
15. Pipetmen (2, 10, 20, 100, 200, and 1000 µL).
16. 15-mL polypropylene conical tubes (Beckton Dickenson, Oakville, Canada; cat. no. 2097).
17. 100 mL 100 m*M* sodium pyruvate (100X; Invitrogen, cat. no. 11360-070).
18. 0.2-µm syringe filters (VWR, Mississauga, Canada; cat. no. 28196-089).
19. 5-cc syringes (Becton Dickenson, cat. no. 309603).
20. Tabletop centrifuge.
21. Polystyrene tissue culture dishes (35, 60, and 100 mm; Corning, Corning, NY; cat. no. 25000-35, 25010-60, and 25020-100, respectively).

22. 500 mL 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (1X; Invitrogen, cat. no. 25200-072).
23. 20 mL type I collagen solution from calf skin (Sigma-Aldrich, Oakville, Canada; cat. no. C-8919).
24. 37°C water bath.

2.1.1. Media

1. 15% DMEM: DMEM is supplemented with 15% heat-inactivated FBS, 1% sodium pyruvate, 1% NEAA, and 1% penicillin-streptomycin. To a 500-mL bottle of DMEM, add 90 mL heat-inactivated FBS, 6 mL penicillin-streptomycin, 6 mL NEAA, and 6 mL sodium pyruvate.
2. KI2: KGM is supplemented with 1% ITS and 2% heat-inactivated FBS. For 100 mL, combine 97 mL KGM, 1 mL ITS, and 2 mL heat-inactivated FBS.

2.1.2. Cells

1. R1 ES cells ***(4)*** (kindly provided by Dr. Janet Rossant and Dr. Andras Nagy, University of Toronto, Toronto, Canada).
2. $K14^{-/-}$ keratinocytes ***(5)***.

2.2. Immunofluorescence

1. 25 g 4-diazabicyclo[2. 2. 2]octane (DABCO) (Sigma-Aldrich, cat. no. D-2522).
2. Goat antirabbit immunoglobulin G, $F(ab')_2$ fragment specific, conjugated to fluorescein isothiocyanate (Cedarlane, Hornby, Canada; cat. no. 111-096-047).
3. Humidified chamber (*see* **Note 3**).
4. K14 antibodies (gift of Dr. Elaine Fuchs, Rockefeller University, New York, NY).
5. Methanol (stored at –20°C).
6. 500 g Mowiol 4-88 (Polysciences Inc., Warrington, PA; cat. no. 17951): for 20 mL, combine 2.4 g Mowiol 4-88 with 6 g glycerol and stir for 2 h at room temperature. Add 6 mL dH_2O and stir for 2 h at room temperature. Add 12 mL 0.2 *M* Tris-HCl at pH 8.5 and heat to 50°C for 10 min with occasional mixing. Using a disposable pipet, transfer the solution to a centrifuge tube and spin at 5000*g* for 15 min. Add 2.5% DABCO to reduce fading. Stir to dissolve. Aliquot into 1-mL tubes and store at –20°C. As needed, a tube is thawed and may be stored for 2 wk at 4°C.
7. 1X PBS (*see* **Subheading 2.1.**, **item 13**).
8. 0.2 *M* Tris-HCl at pH 8.5.

3. Methods

The isolation and maintenance of embryonic fibroblasts (EFs) as well as general protocols for the freezing and thawing of EFs, ES cells, and MKC cells was previously described ***(3)***.

3.1. Mouse Keratinocyte Cells

The one-step culturing program for the differentiation of ES cells along the epidermal lineage described here relies on the expression of K14 as an indicator of successful differentiation. Thus, our co-culture system requires the use of a mature keratinocyte cell line null for K14 to faithfully assay for K14 expression of differentiating ES cells. We therefore use a mitotically inactivated $K14^{-/-}$ mouse keratinocyte cell line isolated from the back skin of K14 knockout mice ***(5)***.

3.1.1. Collagen-Coated Plates

1. Apply 100 µL collagen type I solution to 60-mm tissue culture plates and add 2 mL 1X PBS.
2. Swirl the plate to distribute the collagen solution evenly and allow the solution to coat the plates overnight at 37°C and 5% CO_2.
3. Wash the dishes with three changes of 1X PBS; take care not to damage the coating.
4. After the last PBS wash, dispense 4 mL KI2 medium into the dish and return it to the incubator until the trypsinized cell suspension is prepared (*see* **Subheading 3.1.2., step 6**).

3.1.2. Routine Culture of MKCs

1. Remove the medium from a confluent 60-mm MKC dish and apply 2 mL 0.25% trypsin-EDTA.
2. Agitate the dish back and forth and side to side to distribute the trypsin evenly and return the plate to the incubator for 2–3 min.
3. When the cells detach with gentle agitation, wash the plate with 3 mL KI2 medium and collect the cells into a 15-mL conical tube.
4. Centrifuge for 2–3 min at 700*g*.
5. Aspirate the medium and resuspend the pellet in 5 mL KI2 medium.
6. Plate 1 mL (approx 2×10^5 cells) per 60-mm collagen-coated plate (*see* **Subheading 3.1.1.**) to make a 1:5 dilution of the original culture.
7. Refresh KI2 medium every 2 d.
8. Subculture after 3–4 d. After 2–3 d, the cells can be trypsinized and used to set up for MKC co-cultures (*see* **Subheading 3.2.2.**).

3.2. MKC Co-Culture Preparation

3.2.1. Collagen-Coated Cover Slips

1. Place a 22 × 22-mm cover slip into the bottom of 35-mm tissue culture dishes.
2. Apply 50 µL collagen type I solution to the cover slip and add 1 mL 1X PBS.
3. Agitate back and forth and side to side and coat plates overnight at 37°C and 5% CO_2.
4. Wash the plates three times with 1X PBS and fill each 35-mm plate with 1 mL KI2 medium.
5. Return the dishes to the incubator until the cell suspension is ready (*see* **Subheading 3.2.2., step 6**).

3.2.2. Co-Culture Plate Preparation

1. Trypsinize MKCs in the exponential stage of growth by removing the growth medium and replacing it with 2 mL 0.25% trypsin-EDTA.
2. Agitate the dish back and forth and side to side and return it to the incubator for 2–3 min.
3. Once the cells start to detach, pipet up and down several times with 3 mL KI2 medium to obtain a single-cell suspension.
4. Transfer the cell suspension to a 15-mL conical tube and remove 100 µL for counting.
5. Pellet the cells in the centrifuge for 2–3 min at 700*g*. Meanwhile, use a cell counter to determine the total cell number.
6. For ease of plating, prepare a cell concentration of 10^5 cells/mL in KI2 medium and seed 10^5 cells (1 mL) onto each of the prepared 35-mm dishes (*see* **Subheading 3.2.1., step 5**).
7. Incubate overnight to provide adequate cell attachment before use in co-culture (*see* **Subheading 3.4.**).

3.3. ES Cells

3.3.1. Mitotic Inactivation of EF Feeder Layers

1. Apply 250 µL mitomycin C to a confluent 100-mm EF plate containing 10 mL medium.
2. Mix back and forth and side to side and incubate for 2–3 h at 37°C and 5% CO_2.
3. Wash the dishes with three changes of 1X PBS and fill the plates with 10 mL 15% DMEM (*see* **Note 4**).
4. Return the plates to the incubator until the ES cell suspension is prepared (*see* **Subheading 3.3.2.**, **step 4**).

3.3.2. Routine Culture of ES Cells

1. Rinse a subconfluent dish of ES cells with 1X PBS and trypsinize with 2 mL 0.25% trypsin-EDTA.
2. Agitate the plate back and forth and side to side and place it in the incubator.
3. After 1–2 min, make a single-cell suspension by pipetting up and down 30 times with a 1-mL Pipetman.
4. Make a 1:10 dilution of the original plate by seeding 200 µL (approx 2.5×10^6 cells/100-mm dish) to each prepared feeder layer (*see* **Subheading 3.3.1.**).
5. Distribute the cells evenly and incubate at 37°C and 5% CO_2.
6. Refresh the medium every 2 d.
7. Subculture subconfluent ES cells every 3–4 d (*see* **Note 5**). Generally, ES cells at the point of subculture can be used for differentiation along the epidermal lineage via co-culturing (*see* **Subheading 3.4.**).

3.4. One-Step Differentiation of ES Cells in Co-Culture

ES cells were seeded in co-culture at various cell densities (10^6, 0.5×10^6, 2.5×10^5, 10^5, 5×10^4, and 10^4 cells/35-mm dish) to assay the role of cell density in the commitment of ES cells along the epidermal cell fate. The greatest efficiency was observed in ES cells plated at low density (5×10^4 to 2.5×10^5 cells/35-mm dish) in which small clusters of K14-positive cells were observed after 3 d in culture (*see* **Fig. 1**).

3.4.1. Mitotic Inactivation of MKCs

1. Using the 35-mm plates prepared for co-culture (*see* **Subheading 3.2.2.**), add 50 µL mitomycin C solution to the 2 mL medium in the dishes.
2. Distribute the mitomycin C evenly and incubate for 2–3 h at 37°C and 5% CO_2.
3. Wash the plates three times with 1X PBS (*see* **Note 4**).
4. Fill the plates with 1 mL 15% DMEM and return them to the incubator until the ES cell suspension is prepared (*see* **Subheading 3.4.2.**, **step 7**).

3.4.2. Co-Culture of ES Cells

1. Rinse a subconfluent 100-mm dish of ES cells with 10 mL 1X PBS.
2. Apply 2 mL 0.25% trypsin-EDTA and agitate the dish for even distribution.
3. Return the plate to the incubator for 1–2 min until the cells detach.
4. Pipet up and down 30 times with a 1-mL Pipetman to make a single-cell suspension.
5. Remove a 100-µL aliquot and determine the cell concentration using a cell counter.
6. Using serial dilutions, prepare various concentrations of cells from 10^3 to 10^6 cells per milliliter.
7. Seed 1 mL of various concentrations onto pre-prepared co-culture dishes (from **Subheading 3.4.1.**) (*see* **Note 6**).

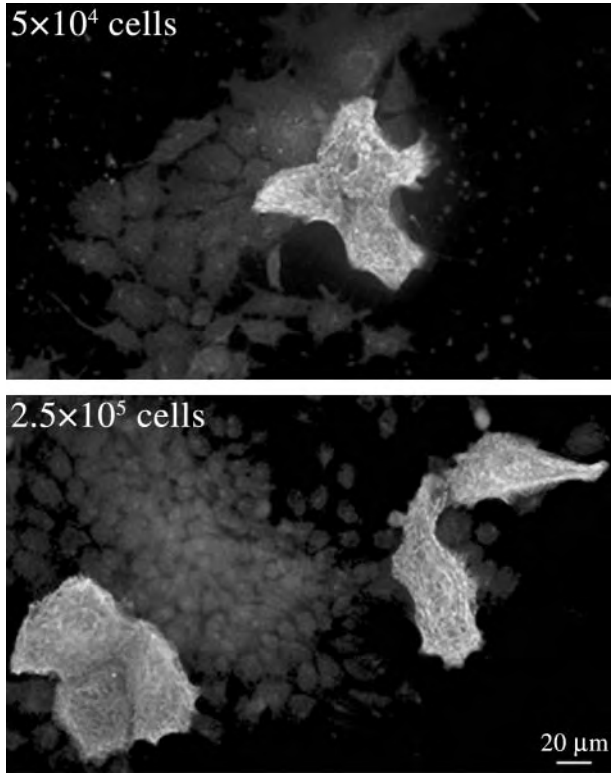

Fig. 1. Embryonic stem cells co-cultured at low density with mitotically inactivated mouse keratinocyte cells develop small clusters of keratin 14-positive cells after 3 d in culture.

8. Gently agitate the dish back and forth and side to side to distribute the cells evenly and incubate at 37°C and 5% CO_2.
9. Refresh the medium every 2 d.

3.5. Immunohistochemical Analysis of Differentiation

1. Remove medium from the co-culture dishes at various time points and fix the cells for 10 min at –20°C with ice-cold methanol.
2. Wash the cover slips three times with 1X PBS.
3. Incubate the cover slips in a humidified chamber for 30 min with 100 µL K14 antibodies (diluted 1:100 in 1X PBS).
4. Wash the cover slips in three changes of 1X PBS.
5. Incubate the cover slips with 100 µL antirabbit secondary antibodies (diluted 1:50 in 1X PBS) at room temperature for 30 min in a humidified chamber.
6. Wash the cover slips three times with 1X PBS.
7. Remove excess PBS from the back of the cover slips with a tissue.
8. Apply 30 µL Mowiol 4-88 on each mounting slide and invert the cover slips onto the slides; take care not to create bubbles.
9. Observe under fluorescence and photograph.

4. Notes

1. Heat inactivate serum in a 57°C water bath for 30 min, mixing every 10 min. Under sterile conditions, aliquot into 100-mL bottles and store at –20°C for 6–12 mo.
2. To thaw for use, wrap the tube in foil to protect the mitomycin C from light and warm in a 37°C water bath. Be careful when handling mitomycin C; it is toxic.
3. To prepare a humidified chamber for immunofluorescence, place a wet piece of filter paper in a 15-cm glass dish with a lid.
4. When washing the mitomycin C-treated cells, extreme caution should be taken not to damage the layer of cells. Aspirate and expel medium and PBS on the sides of the dish rather than directly onto the cells.
5. It is important not to keep ES cells in culture for long periods to maintain pluripotency. Extensive culturing will result in abnormal karyotypes and inconsistent differentiation. Therefore, we generally maintain ES cells in culture only to the sixth passage.
6. The total volume of medium per 35-mm dish should be no more than 2 mL. Greater volumes lead to splashing along the lid and increased contamination potential.

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8

In Vitro Generation of T Lymphocytes From Embryonic Stem Cells

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Summary

Mature hematopoietic cells, like all other terminally differentiated lineages, arise during ontogeny via a series of increasingly restricted intermediates. Hematopoietic progenitors have their origin in the mesoderm, which gives rise to hemangioblasts that can differentiate into endothelial or endocardial precursors or hematopoietic stem cells. These hematopoietic stem cells in turn may either self-renew or differentiate into lineage-restricted progenitors and ultimately mature effector cells. The ability to generate most hematopoietic lineages in a two-dimensional environment in vitro has facilitated our study of this complex process. Until recently, the T-lymphocyte lineage was the exception and appeared to require the specialized three-dimensional microenvironment of the thymus to develop. However, here we describe a protocol for the generation of T lymphocytes from embryonic stem cells in vitro, within a two-dimensional microenvironment, provided by OP9 bone marrow stromal cells. This procedure will facilitate further study of early T lymphopoiesis by providing a simple model system in which the effects of genetic and environmental manipulations of embryonic stem cell-derived progenitors can be examined without requiring other more complex in vivo or in vitro experimental approaches.

Key Words: Delta-like-1; embryonic stem cells; Flt3L; hematopoiesis; IL-7; lymphocyte development; Notch; OP9 stromal cells; stromal cells; T cell development.

1. Introduction

Of the cells in the vertebrate hematopoietic system, the T and B lymphocytes, which comprise the effectors of the adaptive immune system, are arguably the most complex in their development. Both require an inductive environment and must pass developmental checkpoints that allow only cells with functional, nonautoreactive antigen receptors to survive to maturity. Two approaches have successfully generated lymphocytes from embryonic stem cells (ESCs), the totipotent cells isolated from the inner cell mass of a blastocyst *(1)*. In the first, ESCs were differentiated in vitro into three-dimensional

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embryoid bodies (EBs) containing hematopoietic progenitors and then adoptively transferred into recipient hosts to complete their maturation *(2–4)*. This approach produces both T and B cells and is ideal for studying questions of engraftment, homing, and migration in the in vivo context but does not distinguish between defects in potential, survival, or homing. This system also does not allow identification of the maturational intermediates, although recent work indicated that the reconstitution potential of EBs initially resides within the $CD117^{+}CD45^{+}$ population *(5)*.

Although these protocols produce T cells from ESCs, they do not greatly facilitate the manipulation of the hematopoietic environment and progenitors required to gain thorough understanding of the process itself. Thus, further information can be gained from a second approach, which allows ESC differentiation into lymphocytes wholly in vitro. Nakano et al. demonstrated that the in vitro differentiation of ESCs into lymphocytes could be achieved using the bone marrow stromal cell line OP9 *(6,7)*, which is derived from mice deficient in macrophage colony-stimulating factor. The absence of macrophage colony-stimulating factor prevents macrophages from overwhelming other lineages in the co-culture *(8)* and allows OP9 stromal cells to support the differentiation of ESCs (ESC/OP9 co-culture) into multiple hematopoietic lineages, including B lymphocytes.

Although initially the efficiency of B cell generation in ESC/OP9 co-cultures was low, Cho et al. demonstrated that the addition of exogenous Flt3L and IL-7, cytokines known to potentiate B cell production *(9–14)*, allowed for the efficient and consistent production of B cells *(15)*. T-cell potential, however, remained elusive, although transfer of prehematopoietic $Flk\text{-}1^{+}CD45^{-}$ precursors from the ESC/OP9 co-cultures to reaggregate thymic organ cultures, although inefficient, did allow the production of mature T cells from ESCs in vitro *(16)*. Ultimately, the issue of efficiency of in vitro T-cell development was resolved by transducing OP9 stromal cells with the Notch ligand Delta-like-1 (Dll-1) *(17)*.

The Notch signaling pathway had already been demonstrated to play an important role in commitment to the T-vs-B lymphocyte fate *(18,19)*, and when OP9 stromal cells were retrovirally transduced to express Dll-1 (OP9-DL1), the resulting ESC/OP9-DL1 co-cultures now supported robust T, but no longer B, lymphopoiesis from fetal liver, bone marrow, and ESC-derived hematopoietic progenitors *(17,20)*.

This system permits the efficient generation of mature, functional $CD8^{+}$ T cells, although mature $CD4^{+}$ T cells do not arise, presumably because the absence of MHC II on OP9 cells precludes their positive selection. The ESC/OP9-DL1 co-cultures permit detailed molecular and genetic studies under various culture conditions and facilitate the manipulation of ESCs throughout the stages of differentiation from progenitor to mature lymphocyte.

2. Materials

2.1. Cellular Components

2.1.1 ESCs and EF Cells

1. ESCs (R1, D3, E14K derived from 129/Sv mice and ESCs derived from BALB/c and C57BL/6, and [B6x129]F1 mice have all been used to generate lymphocytes in vitro).
2. Mouse embryonic fibroblast (EF) cells *(21)*.
3. Fetal bovine serum (FBS): different sources are required for ESC vs OP9/co-culture medium. For the maintenance of undifferentiated ESCs, Hyclone (Logan, UT) offers

prescreened, characterized lots of FBS. Heat inactivate FBS (iFBS) at 56°C for 30 min and store at 4°C (*see* **Note 1**).
4. High-glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma, Oakville, Canada; cat. no. D-5671). Store at 4°C.
5. 1X phosphate-buffered saline (PBS) without Ca^{2+}/Mg^{2+} (Gibco, Burlington, Canada; cat. no. 14190-144). Store at room temperature.
6. HEPES, sodium pyruvate, gentamicin (HSG) solution: 5 mL 100X or 1 *M* HEPES (Gibco, cat. no. 15630-080); 5 mL 100X or 100 m*M* sodium pyruvate (Gibco; cat. no. 11360-070); and 0.5 mL 1000X or 50 mg/mL gentamicin (Gibco, cat. no. 15750-060). Aliquot into 14-mL conical tubes. Store at 4°C; stable for approx 1 yr.
7. Penicillin-streptomycin, Glutamax, 2(β)-mercaptoethanol (PG2) solution: 5 mL 100X penicillin-streptomycin or 10,000 U/mL penicillin and 10,000 µg/mL streptomycin (Gibco, cat. no. 15140-122); 5 mL 100X or 200 m*M* Glutamax (Gibco, cat. no. 35050-061); 0.5 mL 1000X or 55 m*M* β-mercaptoethanol (Gibco, cat. no. 21985-023). Aliquot into 14-mL conical tubes. Store at –20°C; stable for approx 1 yr.
8. ESC medium: 500 mL high-glucose DMEM supplemented with 15% iFBS, 10.5 mL PGS solution, and 10.5 mL HSG solution (one aliquot each).
9. 2.5% trypsin (Gibco, cat. no. 15090-046): dilute with PBS to 0.25% solution as needed and store at 4°C.
10. Mitomycin C solution: make a 1-mg/mL (100X) mitomycin C (Sigma, cat. no. M-4287) stock solution in PBS. Store in the dark at 4°C; stable for 2 wk.
11. Mouse leukemia inhibitory factor (LIF) (Sigma, cat. no. L5158). Dilute to 7.5 µg/mL (1000X). Aliquot and store at –80°C.
12. Freezing medium: 90% iFBS and 10% dimethyl sulfoxide.
13. Tissue culture ware: tissue culture treated 10- and 6-cm diameter plates; 14- and 50-mL conical tubes; and 5- and 10-mL pipets (suggested supplier Sarstedt, Montreal, Canada).
14. 1.5-mL cryovials (Nalgene Nunc, Rochester, NY).
15. 70-µm nylon mesh cell strainers (BD Biosciences).
16. Refrigerated centrifuge, Allegra 6R (Beckman Coulter, Fullerton, CA).

2.1.2. OP9-DL1 Cells

1. OP9 cells (Riken cell repository, http://www.rtc.riken.go.jp) retrovirally transduced to express Dll-1, as previously reported ***(17)***.
2 α-Modified Eagle's medium (αMEM) (Gibco, cat. no. 12561-056). Store at 4°C.
3. OP9 medium: 500 mL αMEM supplemented with 20% iFBS and 5 mL penicillin-streptomycin.

2.2. ESC/OP9-DL1 Co-Culture

1. Mouse interleukin (IL) 7 (R&D, Minneapolis, MN; cat. no. 407-ML): reconstitute at 1 µg/mL (1000X). Aliquot and store at –80°C.
2. Mouse IL-15 (Peprotech, Ottawa, Canada; cat. no. 210-15). Reconstitute at 25 µg/mL (1000X). Aliquot and store at –80°C.
3. Human Flt3L (R&D; cat. no. 308-FK). Reconstitute at 5 µg/mL (1000X). Aliquot and store at –80°C.

3. Methods

The methods described outline (1) the maintenance of the required cell lines and (2) the co-culture of ESCs on OP9-DL1 cells for the production of T cells or natural killer (NK) cells. It should be noted that all incubations are performed in a standard, humidified, cell

culture incubator at 37°C in 5% CO_2; all tissue culture ware is tissue culture treated for adherent cells, and cells are pelleted by centrifuging at 500*g* for 5 min at 4°C.

3.1. Cellular Components of Co-Culture System

3.1.1. ESCs and EF Cells

3.1.1.1. Preparation of Feeder Layer

ESCs are maintained as adherent colonies on monolayers of growth-inactivated EF cells in ESC medium. EF cell inactivation may be performed by either irradiation (3000 cGy) or treatment with mitomycin C.

1. Incubate EF cells for 2.5 h in ESC medium with 10 µg/mL mitomycin C (*see* **Note 2**).
2. Wash three times with PBS and add fresh ESC medium. EF cells should be used within 5 d of either treatment.

3.1.1.2. Thawing ESCs

1. Thaw ESCs in a 37°C water bath and transfer to a 14-mL conical tube containing 10 mL ESC medium.
2. Pellet the cells and resuspend in 3 mL ESC medium.
3. Plate on a 6-cm dish of approx 80% confluent inactivated EF cells.
4. Add 3 µL LIF.
5. Change the medium the next day and passage to a fresh plate of inactivated EF cells the following day (*see* **Subheading 3.1.1.3.**), each time adding LIF. Maintain ESCs by repeating this procedure, alternating medium changes and passages, and allowing them to become no more than 80% confluent.

3.1.1.3. Passaging ESCs

1. Remove the medium and wash the dish gently with 4 mL PBS.
2. Remove PBS and incubate the plate with 1 mL 0.25% trypsin for 5 min.
3. Wash the cells from the plate by adding 2 mL ESC medium and pipetting vigorously.
4. If the plate has become overconfluent or large colonies with borders of flattened, nonrefractive cells have formed, then small, undifferentiated colonies can sometimes be restored by passing the cells through 70-µm nylon mesh.
5. Pellet cells and resuspend in 3 mL ESC medium with LIF.
6. Remove the medium from a fresh 6-cm dish of 80% confluent inactivated EF cells and add the resuspended ESCs.
7. Gently swish the plate to disperse the cells and LIF.

3.1.1.4. Freezing ESCs

1. To generate frozen stocks of ESCs, wash with PBS, treat with trypsin, and collect the cells as described in **Subheading 3.1.1.3.**
2. Resuspend the ESCs in ice-cold freezing medium and aliquot them into cryovials (two to four vials per confluent 6-cm plate of ESCs).
3. Transfer the vials on ice to a –80°C freezer overnight and the next day to liquid nitrogen for long-term storage.

3.1.2. OP9-DL1 Cells

1. Thaw a vial of OP9-DL1 cells as described for ESCs but substitute OP9 medium for ESC medium.
2. Plate cells in a 10-cm dish with 8–10 mL fresh OP9 medium.

3. Change the medium the next day. OP9-DL1 cells should not be allowed to become more than 80% confluent and can generally be maintained by splitting 1:4 every 2 d.
4. To passage OP9-DL1 cells from a 10-cm plate, remove the medium, wash with 6 mL PBS, remove PBS, and incubate for 5 min with 4 mL 0.25% trypsin.
5. Following trypsin treatment, prepare a 50-mL conical tube with 5 mL OP9 medium.
6. Add 4 mL PBS to the trypsin-treated plate, pipet vigorously, and add the cells to the tube containing medium.
7. OP9-DL1 cells, especially early-passage cells, are very adherent. Rinse the plate again with 8 mL PBS and pool this with the first wash.
8. Pellet the cells, resuspend them, and divide them among four 10-cm plates or four six-well plates.
9. Gently swish the plate to distribute the cells evenly (*see* **Notes 3** and **4**).

3.2. ESC/OP9-DL1 Co-Culture

The protocol for the differentiation of T and NK cells from ESCs is described in **Subheading 3.2.1.** This subheading describes the preparation of the cells for co-culture and the production of T cells and NK cells.

3.2.1. Co-Culture (see ***Note 5***)

3.2.1.1. Days –6 to –2

1. Thaw the ESCs onto inactivated EF cells 4–6 d before beginning the co-culture (d –6 to –4).
2. Maintain undifferentiated ESCs as described in **Subheading 3.1.1.3.**
3. Thaw OP9-DL1 stromal cells at d –4.
4. At d –2, split an 80% confluent plate of OP9-DL1 stromal cells onto four 10-cm plates.

3.2.1.2. Day 0

1. Remove the medium from 10-cm dishes of OP9-DL1 stromal cells that are no more than 80% confluent and replace with 8 mL fresh OP9 medium.
2. Aspirate the medium from the ESCs and treat them with trypsin (*see* **Subheading 3.1.1.3.**).
3. Disaggregate the cells by vigorous pipetting and add 6 mL of ESC medium.
4. Transfer the cells to a new 10-cm dish with no pre-existing EF monolayer.
5. Incubate the cells for 30 min to allow the EF cells to settle and adhere to the plate (plate out).
6. Collect the nonadherent cells from the ESC plate and pellet them.
7. Resuspend the ESCs in 3 mL ESC medium to count.
8. Dilute 5×10^4 ESCs into 2 mL OP9 medium and seed onto a 10-cm dish of 80% confluent OP9-DL1 stromal cells from **step 1**.

3.2.1.3. Day 3

1. Aspirate the co-culture medium without disturbing the cells or the monolayer.
2. Replace with 10 mL fresh OP9 medium.

3.2.1.4. Day 5

At d 5, 50–100% of colonies should have mesoderm characteristics ***(7)*** (*see* **Note 6**).

1. Aspirate the medium without disturbing the cells or the monolayer.
2. Wash with 10 mL PBS and remove the PBS.
3. Add 4 mL 0.25% trypsin to the plates and incubate for 5 min.
4. Disaggregate the cells by vigorous pipetting to create a homogeneous suspension.

5. Add 4 mL OP9 medium and incubate the disaggregated cells for 30 min to plate out the OP9-DL1 cells.
6. Collect the nonadherant cells and pellet them.
7. Resuspend the cells in 2 mL fresh OP9 medium and count them.
8. Seed 6×10^5 cells per fresh 10-cm plate of 80% confluent OP9-DL1 stromal cells. If cells are to be analyzed by flow cytometry at later time points, then a good guideline is to seed one 10-cm plate of OP9-DL1 stroma per anticipated time point (*see* **Note 7**).
9. Add Flt3L to a final concentration of 5 ng/mL.

3.2.1.5. Day 8

Small clusters of 4–10 round, refractile, blastlike cells should be visible.

1. Transfer all the culture medium into a 50-mL conical tube.
2. Gently wash the surface of the plate using a 10-mL pipet with 8 mL PBS; attempt not to disrupt the OP9-DL1 monolayer.
3. Transfer the wash into the same 50-mL conical tube, passing the wash through a 70-µm filter to exclude pieces of disrupted monolayer. The object is to collect all round, loosely adherent, blastlike cells. Check by microscope if this has been accomplished.
4. Pellet the collected cells and resuspend them in 2 mL fresh OP9 medium.
5. Transfer the cells to fresh six-well plates of 80% confluent OP9-DL1 stromal cells: the cells from one 10-cm plate are transferred to one well of a six-well plate in 3 mL of OP9 medium.
6. Add Flt3L to a final concentration of 5 ng/mL.
7. For T-cell differentiation, add IL-7 to a final concentration of 1 ng/mL. For NK cell differentiation, also add IL-15 to a final concentration of 25 ng/mL.

3.2.1.6. Day 10

1. Change medium by collecting culture medium into a 14-mL tube and centrifuging.
2. Add 1 mL fresh OP9 medium to the wells to prevent the cells from drying out.
3. Resuspend any pelleted cells with 2 mL fresh OP9 medium per well of the six-well plate.
4. Gently pipet the resuspended cells onto the original well without disrupting the monolayer.
5. Add cytokines to the final concentrations described in **Subheading 3.2.1.5.**, **step 7**.

3.2.1.7. Day 12

1. Passage the cells by vigorously pipetting to disrupt the monolayer and pass through a 70-µm mesh into a tube.
2. Pellet the cells and resuspend them in 3 mL per well in fresh OP9 medium.
3. Transfer to the same number of wells in fresh six-well plates of 80% confluent OP9-DL1 stromal cells with appropriate cytokines.

3.2.1.8. Beyond Day 12

To continue the cultures beyond d 12, transfer the cells to fresh OP9-DL1 stroma every 4–6 d and change the medium every 2–3 d. Alternate the medium change and passage protocols described for d 10 and 12, respectively. Although for efficient hematopoiesis it is best to leave the co-cultures undisturbed as much as possible, overconfluent OP9-DL1 monolayers no longer support hematopoiesis and may begin to detach from the culture dish and roll up from the edges. Also, note that T cells are sensitive to IL-7 withdrawal and may die should it become exhausted in the culture medium (*see* **Note 7**).

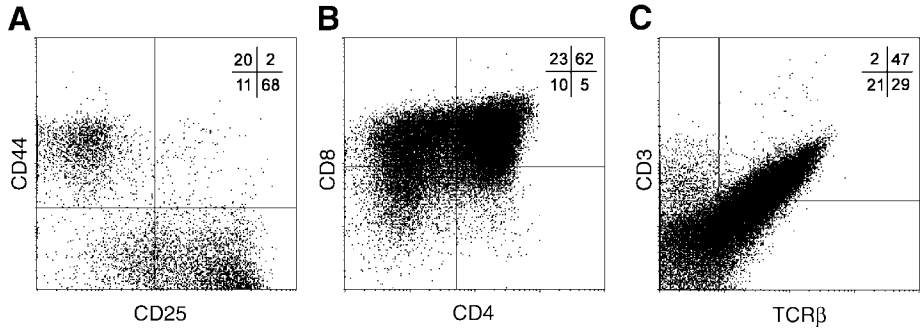

Fig. 1. (**A**) From d 12 to 14, double-negative ($CD4^{-}CD8^{-}$) thymocyte-like populations, characterized by the expression of CD25 and CD44, predominate (a d 14 co-culture is shown). (**B**) By d 16, cells expressing both CD4 and CD8 (double positives) begin to appear. These will predominate by d 20 (a d 24 co-culture is shown). (**C**) αβ-T cells, expressing TCR-β and CD3, appear with similar kinetics as CD4/CD8-expressing cells (a d 22 co-culture is shown). The γδ-T cells generated in this culture system are contained within the $CD3^{+}$ TCR-β^{-} population.

4. Notes

1. Although Hyclone offers prescreened characterized lots of FBS for the propagation of undifferentiated ESCs, prescreened lots of FBS for ESC/OP9-DL1 co-culture are not yet commercially available. To screen FBS for this purpose, co-cultures maintained in OP9 medium supplemented with different lots of iFBS must be run in parallel. The outcome is assessed by the efficiency and cell number of resulting T cells co-expressing the cell surface markers CD4 and CD8 at d 16–20 of co-culture.
2. Mitomycin C is light sensitive.
3. For differentiating ESCs, OP9-DL1 cells should not be kept in continuous culture for longer than 4 wk. OP9-DL1 cells that have been maintained in good condition have a fibroblastic morphology with short dendritic protrusions and an overall starlike shape. OP9-DL1 cells will lose their ability to induce hematopoiesis from ESCs after prolonged culture and allowing overconfluency will hasten this. Noticeably increased or decreased rates of division are indications of OP9-DL1 stroma that may no longer support hematopoiesis from ESCs but may still support hematopoiesis from fetal liver- or bone marrow-derived progenitors. Older stocks of OP9-DL1 cells that may no longer be suitable for initiating an ESC/OP9-DL1 co-culture can still be used at later time points of a co-culture, such as d 8 or 12. During the course of ESC/OP9-DL1 co-cultures, cells are seeded onto 80% confluent OP9-DL1 monolayers, which quickly become overconfluent.
4. To preserve early passage stocks of OP9-DL1 stromal cells, once thawed OP9-DL1 cells have become 80% confluent, split the 10-cm dish into four more dishes and continue until 16 or 32 plates are 80% confluent. Freeze one 80% confluent plate to one cryovial in freezing medium as described for ESCs. These stocks may be expanded to generate working stocks.
5. During the co-culture, hematopoietic cells arise at d 5. Lymphopoiesis, producing DN2 ($CD44^{+}CD25^{+}$) and DN3 ($CD44^{-}CD25^{+}$) thymocytelike cells, may begin as early as d 12 and in the presence of IL-7 and Flt3L should predominate up to d 16 (**Fig. 1A**); thereafter, $CD4^{+}CD8^{+}$-expressing cells should appear (**Fig. 1B**). When co-cultures are maintained past d 20, only lymphoid cells are evident, indicating that multilineage potential does not persist during the co-culture.

6. Mesoderm colonies contain tightly packed refractile cells. Early colonies are flat, and the cells may be arranged in somewhat concentric circles. Later, colonies acquire pronounced three-dimensional structures and resemble asymmetric wagon wheels, with spokes leading out to the rim from a central hub.
7. The kinetics of the co-culture can be assessed by flow cytometry. Hematopoietic cells, defined by the expression of the pan-hematopoietic marker CD45 (leukocyte common antigen) can be detected as early as d 5 of co-culture but more readily by d 8 ***(15)***. Early T-cell progenitors, called DN2 cells, express CD44 and CD25. Expression of CD44 is lost as these progenitors mature to pre-T DN3 cells (**Fig. 1A**). Cells restricted to the T cell lineage can be identified by their expression of CD90 and CD25 and later by upregulation of CD4 and CD8 (**Fig. 1B**) and then surface expression of CD3 and TCR (**Fig. 1C**). In contrast, NK cells express the pan-NK cell integrin CD49b, NKG2D, CD94, and high levels of CD45 but do not express CD24 (HSA) ***(15,22)***. Some NK cells will express low levels of CD45R. It should be noted that cells differentiated from 129-derived ESCs do not express NK1.1, in keeping with that strain's characteristics. Fluorescently labeled antibodies against the markers described can be purchased from either Pharmingen (Mississauga, Canada) or eBiosciences (San Diego, CA).

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9

The Role of *Hex* in Hemangioblast and Hematopoietic Development

Rebecca J. Chan, Robert Hromas, and Mervin C. Yoder

Summary

The orphan homeobox gene *Hex* has been shown to be critical for normal murine liver development using $Hex^{-/-}$ mice. Because of the early lethality of the $Hex^{-/-}$ mice, however, in vitro embryonic stem cell differentiation assays were employed to elucidate the role of *Hex* in murine hematopoiesis. These studies revealed that *Hex* is not required for hemangioblast development but is required in a dose-dependent fashion for hemangioblast differentiation to definitive hematopoietic progenitors. Contained here are the methods needed to conduct embryonic stem cell-derived in vitro hematopoietic assays.

Key Words: Embryonic stem cells; hematopoiesis; Hex; hemangioblasts; in vitro differentiation.

1. Introduction

Hematopoietic and endothelial cells are both derived from mesoderm cells soon after gastrulation in the murine embryo. Evidence has emerged largely from differentiation studies performed using murine embryonic stem (ES) cells that both lineages may be derived from a common precursor cell called the hemangioblast ***(1)***. The stem cell leukemia (*Scl*) and *Runx1* transcription factors as well as vascular endothelial growth factor (VEGF) and its cognate receptor fetal liver kinase-1 (*Flk-1*), have all been demonstrated to play a critical role in the commitment of mesoderm to hemangioblast cells and subsequently to hemangioblast differentiation into hematopoietic or endothelial cell fates ***(2–5)***. At present, little is known about how the expression of these transcription factors and growth factors/receptors is regulated in mesoderm cells leading to the emergence of the hemangioblast and how the levels of these molecules are modulated during hemangioblast proliferation and differentiation.

The Homeobox (*Hox*) gene family has been well recognized as major regulators of multiple aspects of early embryonic development. We and others have previously described the expression of an orphan homeobox gene called *Hex* that is preferentially

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expressed in primitive hematopoietic cells but is downregulated during terminal differentiation of the hematopoietic elements *(4,5)*. *Hex* appears to play many important roles in anterior-posterior organization and is critical for endoderm development. Indeed, homozygous deletion of *Hex* via a targeted deletion in mutant mice results in embryonic lethality caused by severe central nervous system maldevelopment and failure of liver and thyroid development *(6)*.

We have recently determined that *Hex* also plays an important role in hematopoietic development *(7)*. Using $Hex^{-/-}$ ES cells, we demonstrated that *Hex* does not appear necessary for mesoderm commitment to hemangioblast formation or hemangioblast differentiation into primitive erythroblasts. However, there was a dose-dependent requirement for the presence of *Hex* in ES cell-derived embryoid bodies (EBs). Formation of definitive hematopoietic progenitor cells, including burst-forming unit-erythroid (BFU-E) cells, colony-forming unit-granulocyte erythroid macrophage megakaryocyte (CFU-GEMM), colony-forming unit-granulocyte macrophage (CFU-GM), and colony-forming unit-megakaryocyte (CFU-Meg) in EBs was *Hex* dependent. $Hex^{+/+}$ EBs generated from 2.5- to 5.7-fold more definitive progenitors than $Hex^{+/-}$ EBs and 9.5- to 99-fold more definitive progenitors than $Hex^{-/-}$ EBs. Loss of *Hex* expression was also associated with significantly fewer hematopoietic progenitor cells isolated from murine embryonic yolk sacs in vivo. Confirmation of the requirement of *Hex* for normal hematopoietic development was determined in a blastocyst complementation assay in which the contribution of $Hex^{-/-}$ ES cells to all tissues of chimeric mice was assayed. The percentage contribution of $Hex^{-/-}$ ES cells to specific hematopoietic tissues was evaluated by comparing the ratio of the *neo* gene (present in the $Hex^{-/-}$ ES cells) to *Hex* (from the wild-type $Hex^{+/+}$ cells). Whereas the expected ratio of $Hex^{-/-}$ DNA to $Hex^{+/+}$ DNA would be expected to be 1.0 if the ES cells contributed equally to all tissues, $Hex^{-/-}$ DNA was rare in the hematopoietic tissues (<0.25). Thus, our data suggest that *Hex* is not required for the development of the hemangioblast but is required for proper hemangioblast differentiation in vivo and in vitro.

As described, to dissect out the role of *Hex* at various points of hematopoietic differentiation, we utilized ES cell-based in vitro differentiation assays. As the hematopoietic differentiation program within developing EBs has been shown to recapitulate that of yolk sac hematopoiesis during murine development *(8–13)*, this methodology provides a powerful tool to examine the contribution of various gene products to each step of hematopoietic cell differentiation. **Figure 1** shows the overall schema of these assays, which are described in detail in this chapter.

2. Materials

2.1. Equipment and Plasticware

1. 15-mL conical tubes (Corning, Acton, MA; cat. no. 430766).
2. 50-mL conical tubes (Corning; cat. no. 430291).
3. 6- or 10-cm tissue culture plates (BD Falcon, Bedford, MA; cat. no. 35-3002 and 35-3003, respectively).
4. 10-cm Petri dishes (Midwest Scientific, St. Louis, MO; cat. no. 900).
5. 3.5-cm Petri dishes (Midwest Scientific; cat. no. PD351).

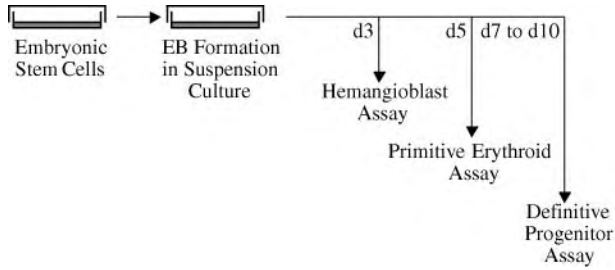

Fig. 1. Schematic diagram outlining the overall embryonic stem cell differentiation process and assessment of various hematopoietic progenitors.

6. 3-cc syringes (Becton Dickinson, Franklin Lakes, NJ; cat. no. 309585).
7. 20-gage, 1.5-in. needles (Becton Dickinson; cat. no. 305176).
8. 16-gage, 1.5-in. needles (Becton Dickinson; cat. no. 305198).
9. 0.22-µm syringe tip filters (Corning; cat. no. 8110).
10. Gelatinized tissue culture plates: to prepare gelatinized tissue culture plates, an adequate volume of gelatin solution (*see* **Subheading 2.2.**, **item 8**) required to completely cover the surface of the tissue culture dish (approx 3 mL for a 6-cm dish and 5 mL for a 10-cm dish) is added and allowed to sit for 20 min at room temperature. The excess gelatin is then aspirated, and the plate is allowed to dry (*see* **Note 1**).
11. Inverted microscope.

2.2. Reagents

1. High-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen Corp., Carlsbad, CA; cat. no. 11965-092).
2. Maintenance fetal bovine serum (FBS) (Hyclone, Logan, UT; cat. no. SH300070.03).
3. 200 m*M* glutamine (StemCell Technologies, Vancouver, Canada; cat. no. 07100).
4. 100X penicillin-streptomycin (StemCell Technologies, cat. no. 07500).
5. 10 m*M* nonessential amino acids (StemCell Technologies, cat. no. 07600).
6. 100 m*M* sodium pyruvate (StemCell Technologies, cat. no. 07000).
7. 5.5×10^{-2} *M* β-mercaptoethanol (1000X; Gibco, Invitrogen, cat. no. 21985-023).
8. Gelatin (StemCell Technologies, cat. no. 07903).
9. Trypsin (StemCell Technologies, cat. no. 07901).
10. Leukemia inhibitory factor (LIF) (10^7 U/mL; Chemicon, Temecula, CA; cat. no. ESG1107).
11. Iscove's modified Dulbecco's medium (IMDM) (Gibco, Invitrogen, cat. no. 12440–053).
12. Differentiation FBS (StemCell Technologies, cat. no. 06900).
13. Holo-transferrin (Sigma, St. Louis, MO; cat. no. T1283).
14. Protein-free hybridoma medium II (PFHM-II) (Gibco, Invitrogen, cat. no. 12040-077).
15. Ascorbic acid (Sigma, cat. no. A4544).
16. Monothioglycerol (Sigma, cat. no. M6145).
17. Methylcellulose (ES-Cult M3120, StemCell Technologies, cat. no. 03120).
18. Stem cell factor (SCF) (Peprotech, Rocky Hill, NJ; cat. no. 250-03).
19. Erythropoietin (Epogen, Amgen, Thousand Oaks, CA).
20. VEGF (Peprotech, cat. no. 450-32).
21. Interleukin (IL)-3 (Peprotech, cat. no. 213-13).
22. Macrophage colony-stimulating factor (M-CSF) (Peprotech, cat. no. 315-02).

23. Granulocyte-macrophage colony-stimulating factor (GM-CSF) (Peprotech, cat. no. 315-03).
24. Plasma-derived serum (Animal Technologies, Antech, TX; cat. no. FBP-186).
25. D4T-conditioned medium (D4T cells were a gift from Dr. Gordon Keller, Mount Sinai School of Medicine, New York, NY).
26. Endothelial cell growth supplement (Fisher Scientific, Pittsburgh, PA; cat. no. BP2654).

2.3. Media

1. DMEM-based ES cell maintenance medium: media used to maintain undifferentiated ES cells in culture includes high-glucose DMEM containing 15% maintenance FBS, 2 m*M* glutamine, 1% penicillin-streptomycin, 1 m*M* sodium pyruvate, 0.1 m*M* nonessential amino acids, 55 μ*M* β-mercaptoethanol, and 1000 U/mL LIF. To prepare medium, start with 500 mL high-glucose DMEM, add 90 mL maintenance FBS, 6 mL each 200 m*M* glutamine, 100X penicillin-streptomycin, 100 m*M* sodium pyruvate, and 10 m*M* nonessential amino acids, 0.614 mL 5.5×10^{-2} *M* β-mercaptoethanol, and 61.4 μL 10^7 U/mL LIF *(7,14,15)*.
2. IMDM-based ES cell maintenance medium: prepare identically to the DMEM-based maintenance medium (**item 1**) with the exception of using IMDM in lieu of high-glucose DMEM *(7,15)*.
3. ES cell differentiation medium: liquid differentiation medium includes IMDM with 15% differentiation FBS, 2 m*M* glutamine, 1% penicillin-streptomycin, 5% PFHM-II, 50 μg/mL ascorbic acid, 200 μg/mL holo-transferrin, and 4.5×10^{-4} *M* monothioglycerol (*see* **Notes 2–4**) *(16)*. To prepare methylcellulose-based differentiation medium, use this recipe and include methylcellulose at a final concentration of 1%. For example, starting with 40 mL 2.3% methylcellulose, add 26.3 mL IMDM; 13.8 mL differentiation FBS; 0.92 mL each 200 m*M* glutamine, 100X penicillin-streptomycin, 5 mg/mL ascorbic acid, and 4.5×10^{-2} *M* monothioglycerol, 4.6 mL PFHM-II, and 3.7 mL 5 mg/mL holo-transferrin.
4. Hemangioblast assay medium: hemangioblast assay medium contains 1% methylcellulose, 10% differentiation FBS, 2 m*M* glutamine, 1% penicillin-streptomycin, 200 μg/mL holo-transferrin, 25% D4T-conditioned medium (*see* **Note 5**), 25 μg/mL ascorbic acid, 4.5×10^{-4} *M* monothioglycerol, 100 ng/mL SCF, and 5 ng/mL VEGF *(1,7,15,16)*. Starting with 40 mL 2.3% methylcellulose, add 13 mL IMDM, 9.2 mL differentiation FBS, 23 mL D4T-conditioned medium, 0.92 mL each 200 m*M* glutamine and 100X penicillin-streptomycin, 0.46 mL 5 mg/mL ascorbic acid, 0.92 mL 4.5×10^{-2} *M* monothioglycerol, 184 μL 50 ng/μL SCF, and 92 μL 5 ng/μL VEGF.
5. Primitive erythroid assay medium: primitive erythroid assay medium contains 1% methylcellulose, 15% plasma-derived serum (*see* **Note 6**), 5% PFHM-II, 2 m*M* glutamine, 1% penicillin-streptomycin, 200 μg/mL holo-transferrin, 50 μg/mL ascorbic acid, 4.5×10^{-4} *M* monothioglycerol, and 5 U/mL erythropoietin *(14,15,17)*. To 40 mL 2.3% methylcellulose, add 0.92 mL each 200 m*M* glutamine, 100X penicillin-streptomycin, 5 mg/mL ascorbic acid, and 4.5×10^{-2} *M* monothioglycerol, 4.6 mL PFHM-II, 3.7 mL 5 mg/mL holo-transferrin, and 230 μL 2 U/μL erythropoietin.
6. Definitive progenitor assay medium: except for the serum and the growth factors, the assay medium for the definitive progenitors is identical to that of the primitive erythroid assay. For definitive hematopoietic assays, differentiation FBS is used rather than the plasma-derived serum. The growth factors are modified depending on the kind of progenitor to be assessed. For definitive erythroid progenitors, add erythropoietin (5 U/mL), SCF (100 ng/mL), and IL-3 (1 ng/mL). For colony-forming unit granulocyte-macrophage (CFU-GM) and colony-forming unit granulocyte erythroid macrophage megakaryocyte (CFU-GEMM), add erythropoietin (5 U/mL), SCF (100 ng/mL), IL-3 (1 ng/mL), GM-CSF (10 ng/mL), and M-CSF (5 ng/mL).

3. Methods

3.1. Murine ES Cell Maintenance

The maintenance of healthy undifferentiated ES cells is critical to the success of the hematopoietic differentiation assays. Included in this subheading are the basic methods needed to sustain ES cells in culture in an undifferentiated state.

3.1.1. ES Cell Plating

ES cells should be maintained in culture only for limited periods of time (up to 3 wk). When maintaining ES cells in culture, they should be grown on gelatinized tissue culture plates in the ES cell maintenance medium as described in **Subheadings 2.1.** and **2.3.** The choice of serum is particularly important as it needs to support ES cell division while maintaining the ES cells in an undifferentiated state (*see* **Note 7**). All ES cell lines grow differently; therefore, it is necessary to determine the growth characteristics of each particular cell line to know the best concentration at which to plate them. For ES cells to grow well and to prevent unwanted differentiation, the cells should be plated to achieve 30–50% confluency (*see* **Note 8**). A good starting point is 500,000 to 1×10^6 cells in a 6-cm plate. The cells can be grown until they reach confluency, and then they should be split.

3.1.2. Splitting ES Cell Cultures

1. Aspirate medium from plate.
2. Add 5–10 mL PBS to wash cells and aspirate.
3. Add 1–3 mL trypsin and place at 37°C for 2–4 min.
4. Add 5–8 mL medium containing at least 10% FBS to quench the trypsin activity. Remove the cells from the plate by pipetting up and down (this trituration can be done using a 5-mL pipet) several times (5–10 times) to break up ES cell clumps (*see* **Note 9**).
5. Use Trypan blue to quantitate cell concentration and plate cells into new gelatinized tissue culture plates to achieve 30–50% confluency.

3.2. Murine ES Cell Differentiation

For primary differentiation, ES cells are plated in differentiation medium (either liquid IMDM based or methylcellulose based) in Petri dishes to prevent ES cell adherence to the plate. When grown in this manner, the ES cells will grow into spheres of cells termed EBs; in this context, these cells have the capacity to undergo differentiation to multiple cell types, including hematopoietic cells, muscle cells, endothelial cells, and neurons. The methods outlined in this chapter are optimized for differentiation to hematopoietic cells. The length of time the EBs should be cultured depends on the type of hematopoietic progenitor to be assessed (**Fig. 1**). For the hemangioblast assay, d 3 EBs are utilized; for the primitive erythroid assay, d 5 EBs are utilized; and for the definitive hematopoietic progenitors, d 7–10 EBs are utilized. EBs cultured 5 d or less can be grown in the IMDM-based liquid differentiation medium. However, for EBs cultured longer than 5 d, the methylcellulose-based differentiation medium is required (*see* **Note 10**).

3.2.1. Plating ES Cells Into Liquid IMDM-Based Differentiation Medium

1. At 24–72 h prior to plating ES cells into differentiation medium, passage the ES cells into IMDM-based maintenance medium.

2. The day that cells are to be plated into primary differentiation medium, trypsinize and quantitate ES cells as described in **Subheading 3.1.2.**
3. Prepare differentiation medium; be sure to add fresh ascorbic acid and freshly diluted monothioglycerol.
4. Plate the ES cells at a concentration of 2000–5000 cells/mL in a total of 9 mL differentiation medium in each 10-cm Petri dish (*see* **Note 11**).
5. Place in a 37°C, humidified chamber with 5% CO_2 for 3–5 d as needed.

3.2.2. Plating ES Cells Into Methylcellulose-Based Differentiation Medium

1. At 24–72 h prior to plating ES cells into differentiation medium, passage the ES cells into IMDM-based maintenance medium.
2. The day that cells are to be plated into primary differentiation medium, trypsinize and quantitate ES cells as described in **Subheading 3.1.2.**
3. Prepare differentiation medium; be sure to add fresh ascorbic acid and freshly diluted monothioglycerol. Mix methylcellulose-based differentiation medium well by vortexing to ensure adequate mixture of medium contents.
4. Aliquot 10 mL differentiation medium into a 15-mL conical tube. Add ES cells at a concentration of 2000–5000 cells/mL and mix well by vortexing.
5. Allow tube to sit at room temperature for 10 min to ensure dissipation of methylcellulose bubbles.
6. Using a 16-gage needle and a 10-mL syringe, draw up 9 mL mixture and distribute to 10-cm Petri dish (*see* **Note 11**).
7. Rock the Petri dish to ensure equal distribution of the medium over the surface of the Petri dish.
8. Place in a 37°C, humidified chamber with 5% CO_2 for 7–10 d as needed.

3.3. Disaggregating EBs Into a Single-Cell Suspension

To conduct each of the progenitor assays, various age EBs are collected and dissociated into a single-cell suspension. As described in **Subheading 3.2.**, for hemangioblasts d 3 EBs are used; for primitive erythroid progenitors, d 5 EBs are used; for definitive progenitors, d 7–10 EBs are used (**Fig. 1**). Following the preparation of the single-cell suspension, the cell concentration is quantitated, and cells are plated out into the progenitor medium for each assay type.

3.3.1. EBs From Liquid IMDM-Based Differentiation Medium

1. Collect d 3–5 EBs using a pipet and place in a 50-mL conical tube. Wash the plate once with 5–10 mL PBS to remove as many EBs as possible. Discard adherent EBs not easily dislodged with the PBS wash.
2. Allow the EBs to settle out of solution by gravity by letting the 50-mL conical tube sit at room temperature for 5 min. Remove and discard the supernatant.
3. Add 10 mL PBS and centrifuge at 300*g* for 5 min., and remove supernatant.
4. Repeat **step 3**.
5. Add 1 mL trypsin and resuspend EBs by shaking tube.
6. Place in a 37°C water bath for 5 min.
7. Add 2 mL serum (either maintenance or differentiation serum) and mix.
8. Using a 3-mL syringe and a 1.5-in. 20-gage needle, disaggregate the EBs by passaging through the needle twice.

9. Centrifuge at 300*g* for 5 min.
10. Resuspend pellet in IMDM plus 10% differentiation serum.
11. Quantitate cell concentration and viability using Trypan blue staining.

3.3.2. EBs From Methylcellulose-Based Differentiation Medium

1. Add 10 mL PBS per 10-cm plate containing 9 mL methylcellulose-based differentiation medium and EBs.
2. Pipet up and down 5–10 times to dilute the methylcellulose into a consistency that can be easily pipeted. Place in a 50-mL conical tube. Wash the plate once with 10 mL PBS to remove as much of the remaining methylcellulose as possible. Discard adherent EBs not easily dislodged with the PBS wash.
3. Centrifuge at 300*g* for 5 min and remove supernatant.
4. Proceed with **steps 4–11** as outlined in **Subheading 3.3.1.**

3.4. Hemangioblast Assay

1. Prepare hemangioblast assay medium as outlined in **Subheading 2.3.**
2. Prepare a single-cell suspension from d 3 EBs as outlined in **Subheading 3.3.1.**
3. Aliquot 4 mL hemangioblast medium to a 15-mL snap-top tube.
4. Add cells to medium (*see* **Note 12**).
5. Vortex mixture well to ensure an adequate distribution of cells throughout the methylcellulose mixture.
6. Allow tube to sit at room temperature for 10 min to allow dissipation of methylcellulose bubbles.
7. Using a 16-gage needle and a 3-mL syringe, draw up 3 mL mixture.
8. Distribute 1 mL mixture to a 3.5-cm Petri dish in triplicate (*see* **Note 13**).
9. Rock the Petri dish to ensure adequate distribution of the mixture over the entire surface of the plate.
10. Place smaller plates in a 20-cm Petri dish with a central well containing water (*see* **Note 14**) and place in a humidified incubator at 37°C with 5% CO_2 for 4 d.
11. Using inverted microscopy, score the number of hemangioblast colonies per plate. Examples of the appearance of a secondary EB and of hemangioblasts are shown in **Fig. 2**.

3.5. Primitive Erythroid Assay

1. Prepare primitive erythroid assay medium as outlined in **Subheading 2.3.**
2. Prepare a single-cell suspension from d 5 EBs as outlined in **Subheading 3.3.1.**
3. Follow **steps 3–9** as outlined in **Subheading 3.4.** (*see* **Note 15**).
4. Place smaller plates in a 20-cm Petri dish with a central well containing water (*see* **Note 14**) and place in a humidified incubator at 37°C with 5% CO_2 for 7 d.
5. Using inverted microscopy, score the number of primitive erythroid colonies per plate. Examples of the appearance of primitive erythroid progenitor colonies are shown in **Fig. 3A**.

3.6. Definitive Hematopoietic Progenitor Assays: Definitive Erythroid, CFU-GEMM, and CFU-GM

1. Prepare definitive progenitor assay medium as outlined in **Subheading 2.3.**
2. Prepare a single-cell suspension from d 7–10 EBs as outlined in **Subheading 3.3.2.**
3. Follow **steps 3–9** as outlined in **Subheading 3.4.** (*see* **Note 15**).
4. Place smaller plates in a 20-cm Petri dish with a central well containing water (*see* **Note 14**) and place in a humidified incubator at 37°C with 5% CO_2 for 7 d.

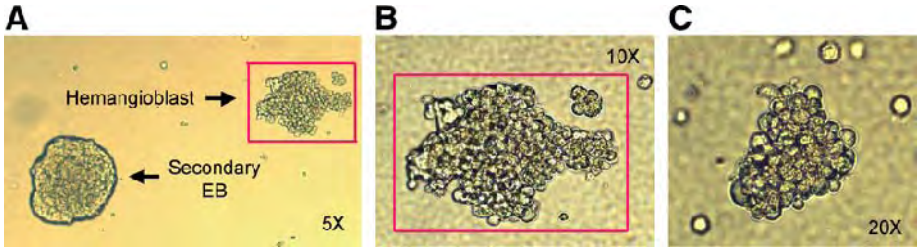

Fig. 2. (**A**) Low-magnification image showing a secondary embryoid body (EB) and a hemangioblast colony in the same field for comparison. Secondary EBs are spheroid and composed of small, nonrefractile cells; the hemangioblast colonies are irregular and composed of larger, refractile cells. (**B**) Higher magnification of hemangioblast colony shown in **A**. (**C**) Another example of a hemangioblast colony. (Please *see* companion CD for the color versions of this figure.)

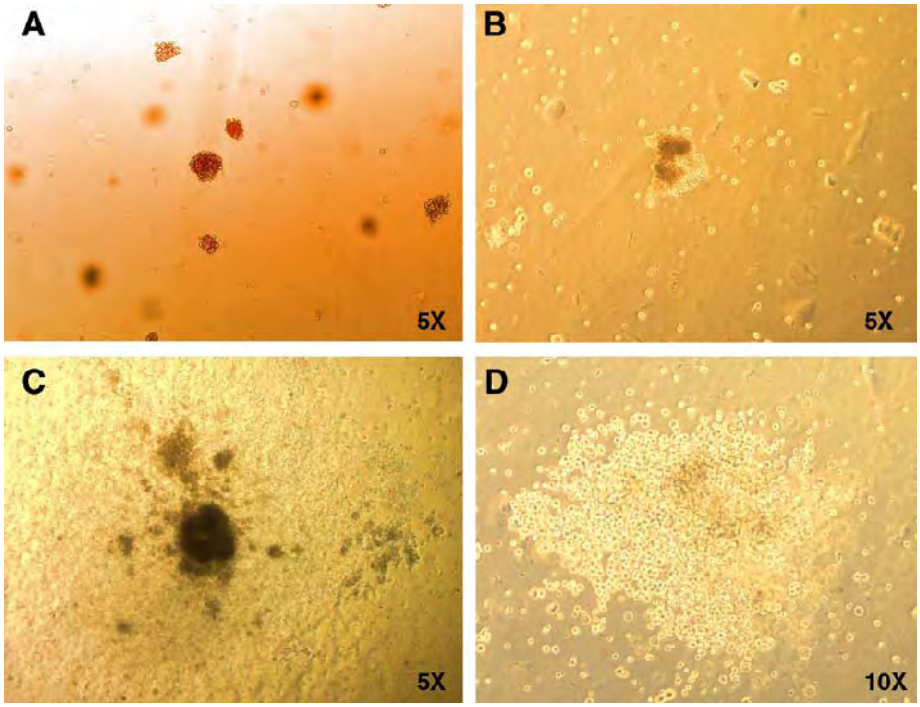

Fig. 3. Images demonstrating morphological characteristics of various progenitor colonies. (**A**) Primitive erythoid colonies are small and composed of bright red, nucleated cells. (**B**) Definitive erythroid colonies are composed of orange-brown, small, enucleated cells. (**C**) CFU-GEMM colonies are composed of both erythroid (dark color cells in center of colony) and myeloid (light color halo of cells in the periphery of the colony) cells. (**D**) CFU-GM colonies are composed purely of myeloid cells, which are light color and refractile. (Please *see* the comapanion CD for the color versions of this figure.)

5. Using inverted microscopy, score the number of definitive erythroid colonies, CFU-GEMM, or CFU-GM per plate as indicated. Examples of the appearance of these various colonies are shown in **Fig. 3B–D**.

4. Notes

1. To allow tissue culture plates to dry, prop the tissue culture plate lid open inside a HEPA-filtered laminar flow biosafety cabinet with the blower running until the liquid component of the gelatin solution has completely evaporated.
2. Ascorbic acid comes in a powered form that is soluble in water. Prepare a working 5 mg/mL concentration, filter-sterilize (0.22-µm filter), aliquot, and store at –20°C in the dark (ascorbic acid is light sensitive). Always add ascorbic acid to medium just before use.
3. Holo-transferrin is dissolved in water to a 5 mg/mL concentration, filter-sterilize (0.2-µm filter), aliquot, and store at –20°C.
4. The monothioglycerol comes in a viscous liquid with a density of 1.25 g/mL. Based on its molecular weight of 108.2 g/mol, prepare the 4.5×10^{-2} *M* working solution by adding 38 µL undiluted monothioglycerol into 10 mL PBS. As the undiluted monothioglycerol is very viscous, caution needs to be taken to allow the full 38 µL to be aspirated into the pipet tip and to ensure that the full volume is delivered into the 10 mL PBS. The diluted 4.5×10^{-2} solution needs to be mixed well, filter-sterilized (0.22-µm filter), and used the day of preparation. *Do not store* the diluted monothioglycerol; it must be diluted fresh for each use. Store the undiluted monothioglycerol in aliquots at –20°C.
5. To prepare D4T-conditioned medium, culture D4T cells on gelatinized tissue culture plates in IMDM with 10% maintenance FBS, 1% penicillin-streptomycin, and 50 µg/mL endothelial cell growth supplement until cells are confluent. Once confluent, remove the medium and add new medium for a total of 72 h. Collect the conditioned medium and filter through 0.45-µm filter to remove cellular debris. Aliquot and store at –80°C. Each batch of D4T-conditioned medium needs to be tested to support hemangioblast growth.
6. The plasma-derived serum is shipped frozen from Animal Technologies. Once thawed, to remove large precipitates, spin at 2060*g* and retain the supernatant. Filter through a no. 1 Whatman paper and then through a 0.45-µm filter using a prefilter. Heat inactivate for 30 min at 56°C. Aliquot and store at –80°C.
7. For maintenance of ES cells, be sure to use ES cell-tested serum. We have consistently had good results using FBS from Hyclone (**Subheading 2.2.**, **item 2**).
8. Although it is important to avoid plating the cells too sparsely, care must also be taken to prevent the cells from overgrowing. When the ES cells become overconfluent, they have the capacity to grow into EB-like structures and to undergo differentiation.
9. It is critical to remove all ES cell clumps prior to replating on new gelatinized plates. When replated, any remaining cell clumps will grow into EB-like structures with an increased capacity to undergo differentiation. If clumping is a problem, then pipetting up and down vigorously using a sterile (autoclaved) glass Pasteur pipet as an alternative method.
10. As the EBs have an increased capacity to adhere to the surface of the plate as they grow and enlarge, the methylcellulose-based differentiation medium is needed for EBs grown longer than 5 d, which helps to prevent EB adherence.
11. The use of the specific Petri dishes listed in **Subheading 2.1.**, **item 4**, is strongly recommended. We have found that the tendency of the EBs to adhere to these plates is less than that of other Petri dishes.
12. As stated, the number of cells needed for optimal plating will vary among ES cell lines. The goal is to have an adequate number of hemangioblast colonies per plate (50–150) without

overgrowth of secondary EBs (EBs that grow within the hemangioblast progenitor assay cultures from contaminating undifferentiated ES cells; **Fig. 2**). A good starting point for this assay is 12,500 cells/mL.

13. It is imperative to use Petri dishes for this step, not standard tissue culture-grade progenitor assay plates. In tissue culture-grade plates, the secondary EBs and the progenitors will adhere to the plate surface rather than remain in suspension, and it is impossible to quantitate the progenitors.
14. Given the small volume of methylcellulose mixture in each plate (1 mL), it is necessary to maintain the progenitor assay plates in a highly humidified chamber to prevent dehydration. This is simply done by placing the progenitor assay plates within a 20-cm Petri dish with one 3.5-cm dish filled with water (lid off) in the center of the 20-cm Petri dish (evaporation of the water from this central dish ensures that the contiguous progenitor plates are hydrated at high humidity).
15. For primitive erythroid assays and definitive hematopoietic progenitor assays, a good cell concentration to start with is 50,000 cells per milliliter.

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10

Generation of Osteoblasts and Chondrocytes From Embryonic Stem Cells

Jitsutaro Kawaguchi

Summary

The efficient generation of mesenchymal cells such as adipocytes, osteoblasts, and chondrocytes from embryonic stem cells is achieved by following sequential steps: embryoid body formation, retinoic acid (RA) treatment, and exposure to specific reagents for differentiation. RA treatment of embryoid bodies is critical for subsequent mesengenesis. Adipogenesis, osteogenesis, and chondrogenesis occur by culturing outgrowths for 2–3 wk with insulin/triiodothyronine, bone morphogenetic protein/dexamethasone-β/glycerophosphate/ascorbic acid, and transforming growth factor-β_3/parathyroid hormone/1% fetal bovine serum, respectively. Emergence of these mesenchymal cells using a common initial procedure suggests that embryoid body formation and subsequent RA treatment results in the generation of a common progenitor for osteoblasts and chondrocytes.

Key Words: Adipocyte; bone morphogenetic protein; chrondrocyte; embryonic stem cell; osteoblast; transforming growth factor-β.

1. Introduction

Osteoblasts and chondrocytes derived from ectoderm and mesoderm make up bone and cartilage elements during embryogenesis and adulthood *(1)*. Neural crest cells, which arise from the dorsolateral edge of the neural plate, give rise to the facial element of the skeleton *(2)*, and sclerotome arising from paraxial mesoderm gives rise to structures called mesenchymal condensations *(3)*. These highly condensed cells differentiate into chondrocytes to form primordial cartilage of vertebrae. Perichondrium surrounding the primordial cartilage is thought to invade the bone marrow and give rise to osteoblasts and mesenchymal stem cells on vascular invasion *(4)*, although the precise mechanism for this is not known.

Mesenchymal stem cells reside in bone marrow throughout adulthood and take on an important role for the supplementation of osteoblasts and chondrocytes for bone remodeling and regeneration of bone on fracture *(5)*. They grow in an immature state on plastic dishes while maintaining the differentiation potential to become adipocytes,

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osteoblasts, or chondrocytes in specific reagents *(6)*. In spite of their physiological and pathological importance, our knowledge of the developmental origin of mesenchymal stem cells and the molecular program that gives rise to immature mesenchymal cells from early embryogenesis is relatively limited.

The formation of osteoblasts and chondrocytes requires multistep differentiation processes. Considering the fact that embryonic stem (ES) cells generally follow the developmental program of embryogenesis during their in vitro differentiation *(7)*, a protocol for the generation of mesenchymal cells from ES cells might be useful to dissect the developmental process and mechanism of mesengenesis through ectodermal and mesodermal formation. Also, it will open the possibility for a role in future pharmaceutical screening or medical treatment. A protocol for the generation of adipocytes from ES cells has been described previously *(8)*. Based on this I have developed a method to induce the mesenchymal lineage from ES cells with relatively high efficiency *(9)*.

2. Materials

2.1. Reagents

1. Absolutely RNA reverse transcriptase polymerase chain reaction (RT-PCR) miniprep kit (Stratagene, La Jolla, CA; cat. no. 400800).
2. Acetic acid (BDH Laboratory Supplies, Poole, UK; cat. no. UN2789).
3. Alcian blue 8GX (Sigma, St. Louis, MO; cat. no. A5268): for alcian blue solution. Dissolve 1 g Alcian blue in 100 mL 0.1 *N* HCl.
4. Alizarin red S (Sigma, cat. no. A5533): for alizarin red solution, preheat 100 mL distilled water (dH_2O) at 45°C. Add 2 g alizarin red. Stir and allow to reach room temperature. Adjust to pH 4.2 by adding a few microliters of NH_4OH (*see* **Note 1**).
5. All-*trans* retinoic acid (RA) (Sigma, cat. no. R2625).
6. Ammonium hydroxide (Sigma, cat. no. A6899).
7. L-Ascorbic acid 2-phosphate, sesquimagnesium salt (AA) (Sigma, cat. no. A8960).
8. BD Plastipak™ 10-mL (10-mL sterile syringe) (Becton Dickinson, Franklin Lakes, NJ; cat. no. 302188).
9. Bovine serum albumin (BSA) (Sigma, cat. no. A9418).
10. 150-mL bottle-top filter, 0.2-µm cellulose acetate membrane (Becton Dickinson, cat. no. 357111).
11. Collagenase/dispase (Roche, East Sussex, UK; cat. no. 269-683).
12. Chick serum (Sigma, cat. no. C5405).
13. Dexamethasone (Dex) (Sigma, cat. no. D8893).
14. Dimethyl sulfoxide (DMSO) (Sigma, cat. no. D8779).
15. 0.5-mL sterile dispenser tip (Alpha Laboratories, Eastleigh, UK; cat. no. DT7301S).
16. Dulbecco's phosphate-buffered saline (PBS) (Sigma, cat. no. D8537).
17. ESGRO 10^6 U (leukemia inhibitory factor, LIF) (Chemicon, Temecula, CA; cat. no. ESG1106).
18. Ethanol (BDH Laboratories Supplies, cat. no. UN1170).
19. Ethylenediaminetetraacetic acid, disodium salt: dihydrate (EDTA) (Sigma, cat. no. E5134).
20. Fetal bovine serum (FBS) characterized and screened for ES cell growth (HyClone, Logan, UT; cat. no. CH30160.03).
21. Formaldehyde solution 37/40% (formalin) (Fisher Scientific, Leicestershire, UK; cat. no. F1500PB15).
22. Gelatin (Sigma, cat. no. G1890).

23. Glasgow minimum essential medium (GMEM) (Sigma, cat. no. G5154).
24. Liquid 200 m*M* L-glutamine (Invitrogen, Carlsbad, CA; cat. no. 25030-123).
25. Glycerol (Sigma, cat. no. G5516).
26. Glycerol 2-phosphate disodium salt hydrate (β-glycerophosphate or β-GP) (Sigma, cat. no. G9891).
27. Guanidine hydrochloride (guanidine HCl) (Fluka, Buchs, Switzerland; cat. no. 50935).
28. Human recombinant bone morphogenetic protein (BMP)-4 (R&D Systems, Minneapolis, MN; cat. no. 314-BP-010).
29. Human recombinant transforming growth factor (TGF)-β_3 (R&D Systems, cat. no. 243-B3-002).
30. 11.6 *M* HCl, specific gravity 1.18 (BDH Laboratory Supplies, cat. no. UN1789).
31. Insulin (Sigma, cat. no. I1882).
32. Isopropyl alcohol (BDH Laboratories Supplies, cat. no. 296942D).
33. Lid for microtiter plate (Sterilin, Staffordshire, UK; cat. no. 642000).
34. LightCycler system (Roche, cat. no. 2 011 468).
35. LightCycler capillaries (Roche, cat. no. 1 909 339).
36. LightCycler-fast start DNA master SYBR green I (Roche, cat. no. 2 239 264).
37. 2-Mercaptoethanol (BDH Laboratories Supplies, cat. no. 441433A).
38. Liquid 10 m*M* minimum essential medium (MEM) nonessential amino acids solution (100X; Invitrogen, cat. no. 11140-050).
39. Flat-bottom, six-well microplate (Iwaki, Tokyo, Japan; cat. no. 3810-006).
40. Flat-bottom, 96-well microplate (Iwaki, cat. no. 3860-096).
41. V-bottom, 96-well microtiter plate (Sterilin, cat. no. 612V96).
42. Oil Red O (Sigma, cat. no. O0625): for Oil Red O solution, dissolve 0.3 g Oil Red O in 100 mL isopropyl alcohol. Prepare a working solution of Oil Red O:dH_2O = 6:4 prior to use.
43. 4% paraformaldehyde (Sigma, cat. no. P6148): preheat 50 mL PBS at 70°C. Dissolve 2 g paraformaldehyde in PBS and incubate for 30 min at 70°C. Store at –20°C.
44. Parathyroid hormone (PTH) fragment 1-34 bovine (Sigma, cat. no. P3671).
45. Triple-vent 90-mm Petri dish (bacteriological dish) (Sterilin, cat. no. 101VR20).
46. Single-use 0.20-µm syringe filter (Sartorius AG, Goettingen, Germany; cat. no. 16534).
47. Sodium hydroxide (NaOH) (Fisher Scientific, cat. no. S/4920/53).
48. Liquid 100 m*M* sodium pyruvate MEM (Invitrogen, cat. no. 11360-88).
49. 25-mL Steripette (disposable serological pipet; Corning, Big Flats, NY; cat. no. 4251).
50. 10-mL Steripette (disposable serological pipet; Corning, cat. no. 4101).
51. 5-mL Steripette (disposable serological pipet; Corning, cat. no. 4051).
52. SuperScript first-strand synthesis system for RT-PCR (Invitrogen, cat. no. 11904-018).
53. 25-cm^2 tissue culture flask (T-25 flask; Iwaki, cat. no. 3100-025).
54. Topo TA cloning kit dual promoter with PCRII-TOPO vector (Invitrogen, cat. no. K4600-01).
55. 3,3,5-Triiodo-L-thyronine (T_3) (Sigma, cat. no. T5516).
56. Liquid trypsin (10X; Invitrogen, cat. no. 15090-046).
57. 30-mL universal container (Sterilin, cat. no. 128A).
58. 1-200-µL yellow pipet tips (sterilize by autoclave before use) (Starlab, Ahrensburg, Germany; cat. no. S1111-0006).

2.2. Tissue Culture (see Note 2)

1. Glutamine and pyruvate solution: mix equal volumes of 200 m*M* L-glutamine and 100 m*M* sodium pyruvate MEM. Store at –20°C.
2. β-Mercaptoethanol: add 200 µL β-mercaptoethanol to 28.2 mL dH_2O. Store at 4°C and use within 1 mo.

3. ES medium: GMEM supplemented with 1X MEM nonessential amino acids, 2 m*M* glutamine, 1 m*M* pyruvate, 100 m*M* β-mercaptoethanol, 10% FBS, and 5X 10^5 U of LIF. To 500 mL GMEM, add 5.5 mL MEM nonessential amino acids, 11 mL glutamine and pyruvate solution, 550 µL β-mercaptoethanol, 55 mL FBS, and 500 µL LIF. Store at 4°C for up to 1 mo.
4. Insulin (10 mg/mL = 1.7 µ*M*): add 100 µL acetic acid to 10 mL dH_2O to prepare acidified distilled water (pH 2.5). Dissolve insulin in acidified distilled water to make 10 mg/mL solution. Store at –20°C.
5. T_3 (20 µg/mL = 0.3 µ*M*): dissolve T_3 in 1 *N* NaOH to make 1 mg/mL solution. Add 50 vol GMEM to prepare a final concentration of 20 µg/mL. Store at –20°C.
6. Adipogenesis medium: ES medium without LIF supplemented with 85 n*M* insulin and 2 n*M* T_3. To 20 mL ES medium without LIF, add 1 µL insulin and 1.4 µL T_3. Reagent is active for 2 wk.
7. 100X β-GP/AA (1 *M* and 17 m*M*, respectively): dissolve 1.1 g β-GP and 25 mg AA in 5 mL PBS. Sterilize by filtration and store at –20°C.
8. 500X Dex (20 µg/mL = 50 µ*M*): dissolve Dex in ethanol to make 1 mg/mL solution. Add 50 vol GMEM to prepare a final concentration of 20 µg/mL. Store at –20°C.
9. 100X BMP-4 (10 µg/mL): dissolve in sterile 4 m*M* HCl containing 0.1% BSA to prepare a final concentration of 10 µg/mL. Aliquot 200 µL each and store at –20°C. Solution is stable at –20°C for 6 mo and at 4°C for 1 mo.
10. Osteogenesis medium: ES medium without LIF supplemented with 10 m*M* β-GP, 170 µ*M* AA, 100 n*M* Dex, and 100 ng/mL BMP-4. To 20 mL ES medium without LIF, add 200 µL 100X β-GP/AA, 40 µL 500X Dex, and 200 µL 100X BMP-4. Reagent is active for 2 wk.
11. 100X TGF-β_3 (1 µg/mL): dissolve in sterile 4 m*M* HCl containing 0.1% BSA to prepare a final concentration of 1 µg/mL. Aliquot 200 µL each and store at –20°C. Solution is stable at –20°C for 6 mo and at 4°C for 1 mo.
12. 100X PTH (10 µ*M*): dissolve 0.1 mg PTH in 2.4 mL 1 m*M* HCl containing 0.1% BSA to a final concentration of 10 µ*M*. Store at –20°C.
13. Chondrogenesis medium: ES medium without LIF and FBS supplemented with 10 ng/mL TGF-β_3, 100 n*M* PTH, and 1% FBS. To 20 mL ES medium without LIF and FBS, add 200 µL TGF-β_3, 200 µL 100X PTH, and 200 µL FBS. Reagent is active for 2 wk.
14. Gelatin: dissolve 2.5 g gelatin in 250 mL dH_2O to make 10X gelatin (1%). Stir with heat and sterilize by autoclave. Store at 4°C. Dilute 10 times with PBS to make 1X gelatin (0.1%).
15. Trypsin: dissolve 0.186 g EDTA in 500 mL PBS to make 1 m*M* EDTA solution. Sterilize EDTA solution by filtration using bottle-top filter and add 5 mL chicken serum and 5 mL trypsin (10X) to make 1% chicken serum/0.1X trypsin. Store at –20°C.
16. RA: dissolve 50 mg RA in 16 mL DMSO to make 10^{-2} *M* solution. Store at –20°C.

3. Methods

The protocol for the derivation of adipocytes, osteoblasts, and chondrocytes from ES cells consists of four major steps: (1) maintenance of ES cells without feeders; (2) embryoid body (EB) formation and subsequent RA treatment in suspension culture; (3) terminal differentiation of cells using specific reagents; and (4) detection of mesengenesis by quantitative RT-PCR and histochemical staining. An overall diagram of the protocol is shown in **Fig. 1B**. In addition, a method to induce mesenchymal cells after dissociation of EBs is introduced.

3.1. Maintenance of ES Cells Without Feeders

1. Incubate T-25 flask with 1X gelatin for 5 min at room temperature (gelatinization).
2. Grow ES cells on gelatin-coated T-25 flask without feeders using ES cell medium (**Fig. 1A**, **left panel**; *see* **Notes 3** and **4**).

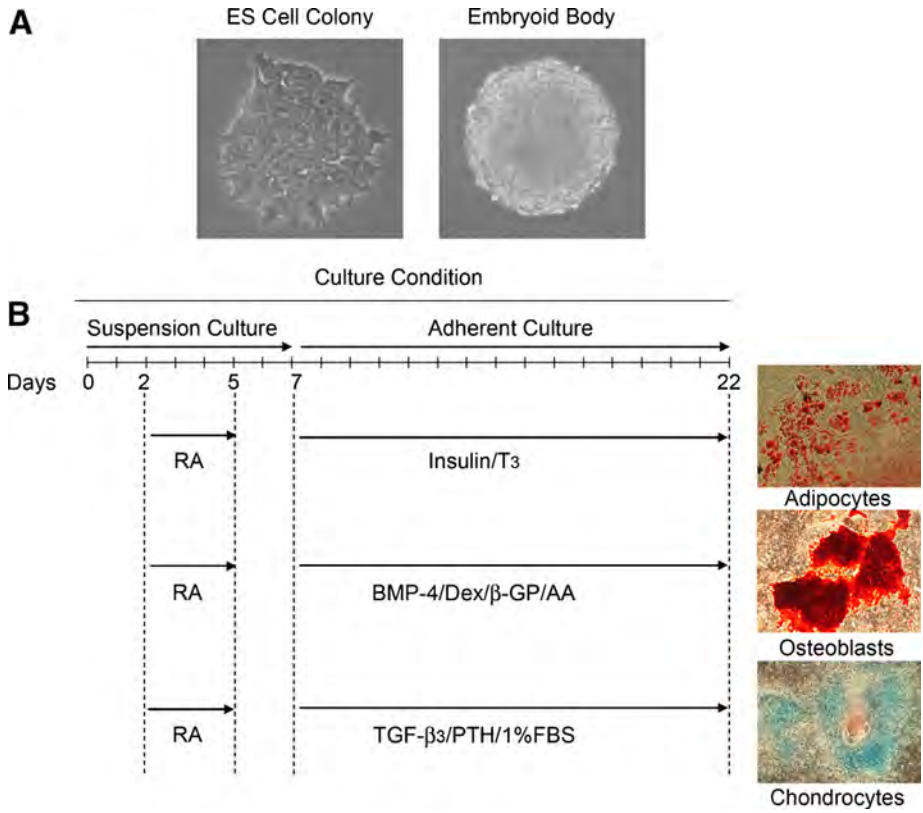

Fig. 1. Schematic representation of mesengenesis from embryonic stem (ES) cells. (**A**) ES cell colony grown without feeders (left); single embryoid body formed by hanging drop method (right). (**B**) The overall differentiation scheme for mesengenesis from ES cells. Typical results of Oil Red O (adipocytes), alizarin red (osteoblasts), and alcian blue staining (chondrocytes) are shown in right panel (Please *see* the companion CD for the color versions of this figure.)

3. Passage cells before they become confluent (*see* **Note 5**).
4. Plate 2–3 million cells in T-25 flask the day before EB formation to minimize number of differentiated cells (*see* **Note 6**).

3.2. EB Formation by Hanging Drop Method (see *Note 7)*

1. Wash cells twice with PBS.
2. Add trypsin and incubate at 37°C for 1 min (*see* **Note 8**).
3. Check separation into single cells after mild tapping of flask.
4. Add medium to stop the reaction.
5. Centrifuge at 260*g* for 3 min and count cells.
6. Dissociate cells in medium and adjust to 10^5 cells/mL.
7. Fill 90-mm bacteriological dish with 20 mL PBS (*see* **Note 9**).
8. Make 10-μL drops (10^3 cells per drop) on the inside of the lid using serial pipet (**Fig. 2A**; *see* **Notes 10** and **11**).

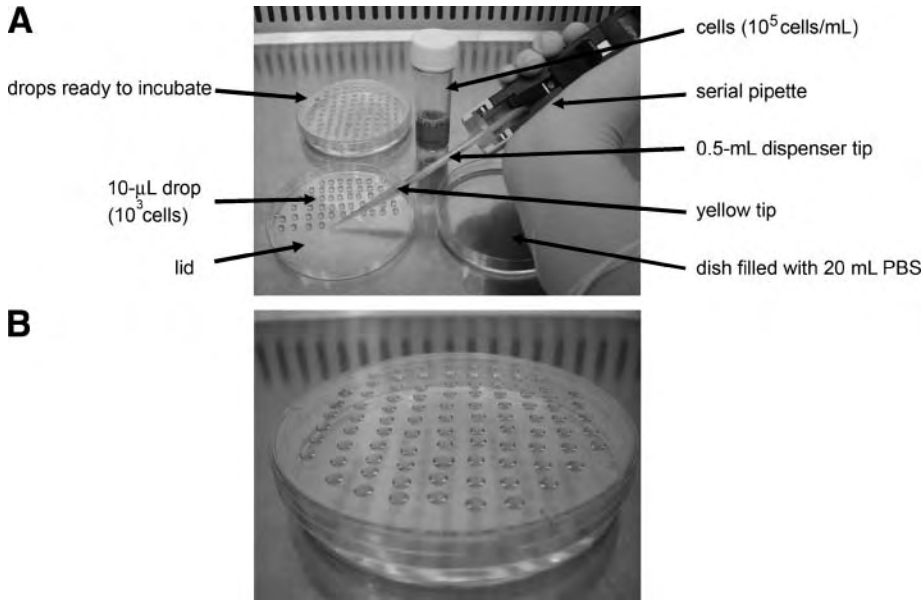

Fig. 2. Preparation of embryoid bodies by hanging drop method. (**A**) Generation of drops. (**B**) Plate with drops on inside of the lid.

9. Carefully turn the lid around and fit it on the dish (**Fig. 2B**).
10. Culture for 2 d (*see* **Note 12**).
11. Check that a single EB is formed on the center of each drop (**Fig. 1A**, **right panel**; *see* **Note 13**).
12. Transfer EBs by bringing the lid down sharply on a 90-mm bacteriological dish containing 10 mL medium. The EBs will fall into the medium in the dish (**Fig. 3**).
13. Thaw RA stock (10^{-2} *M*) and dilute 20 times with DMSO (concentration is 5×10^{-4} *M*). Add 2 µL diluted RA in 10 mL medium to prepare final concentration of 10^{-7} *M* (*see* **Note 14**).
14. Expose EBs to RA for 3 d in culture (*see* **Note 15**).
15. Transfer EBs to a universal; wait until all EBs settle on the bottom.
16. Aspirate medium and add 10 mL fresh medium.
17. Collect EBs and grow them for another 2 d on bacteriological dish without RA (*see* **Note 16**).
18. Prepare gelatin-coated six-well plates by incubating them with 1X gelatin for 5 min at room temperature and fill each well with 1.5 mL medium.
19. Seed 20–30 EBs in each well and allow them to attach to the dish overnight.

3.3. Terminal Differentiation

1. The day after plating, change medium to differentiation medium: adipogenesis (**Subheading 2.1., item 6**), osteogenesis (**Subheading 2.1., item 10**), or chondrogenesis (**Subheading 2.1., item 13**) (*see* **Notes 17** and **18**).
2. Change half of the medium after 6 d and every 3 d thereafter.
3. Check differentiation by quantitative RT-PCR and by histochemical staining at around d 10–18 after plating (*see* **Notes 19–21**).

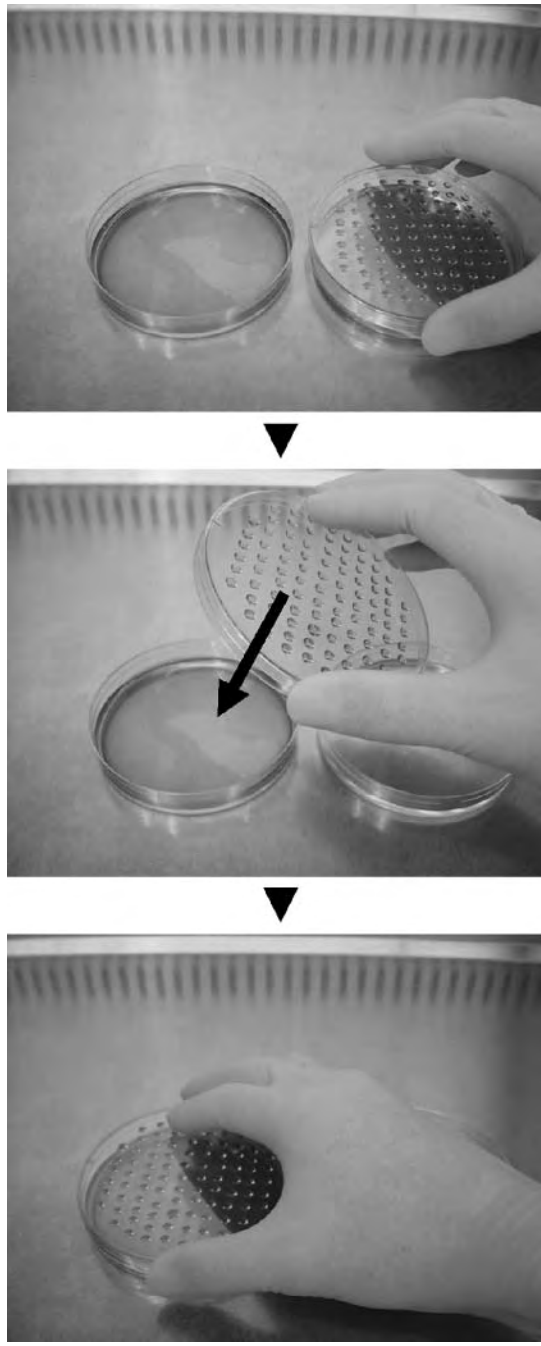

Fig. 3. Sequential image of embryoid body collection.

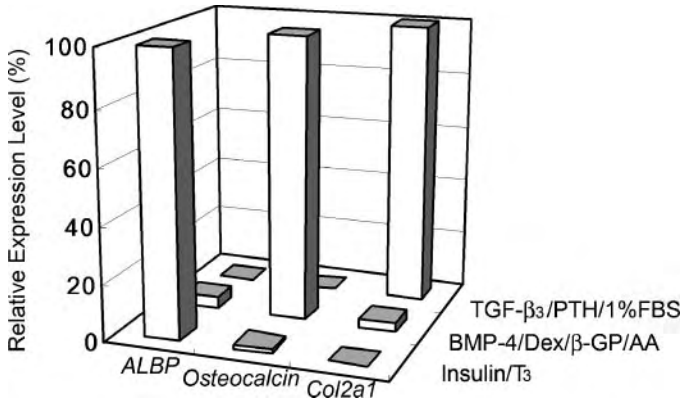

Fig. 4. Adipogenesis, osteogenesis and chondrogenesis in embryoid body (EB) outgrowths. EB outgrowths (CGR8) were cultured with indicated reagent for 18 d and subjected to quantitative reverse transcriptase polymerase chain reaction. Expression level of each gene is normalized against β-*actin*. Highest expression level is defined as 100%.

3.4. Detection of Mesengenesis

3.4.1. Reverse Transcriptase Polymerase Chain Reaction (see ***Note 22***)

As it is not easy to identify the development of mesenchymal cells by morphology except for adipocytes, quantitative RT-PCR is employed to detect differentiation of mesenchymal cells (**Fig. 4**). Primers and PCR conditions for representative markers for each mesenchymal cell type are shown in **Table 1**. Generally, terminal differentiation occurs between 10 and 18 d after plating (*see* **Notes 19–21**).

3.4.1.1. Extraction of RNA (*see* **Note 23**)

1. Wash cells twice with PBS and add 350 µL lysis buffer containing β-mercaptoethanol (2.5 µL).
2. Collect cells and vortex. Samples can be stored at –80°C.
3. Transfer to a prefilter and spin at 16,060*g* for 5 min.
4. Remove cap and add 350 µL 70% ethanol.
5. Transfer to an RNA-binding cap, spin for 30 s, and discard filtrate.
6. Add 600 µL low-salt solution, spin for 30 s, and discard filtrate.
7. Spin the tube at 16,060*g* for 2 min and discard filtrate.
8. Add 5 µL DNase to 50 µL DNase digestion buffer.
9. Add DNase solution onto the fiber matrix.
10. Incubate at 37°C for 15 min in an air incubator.
11. Add 600 µL high-salt wash buffer, spin for 30 s, and discard filtrate.
12. Add 600 µL low-salt buffer, spin for 30 s, and discard filtrate.
13. Add 300 µL low-salt buffer and spin at 16,060*g* for 2 min.
14. Add 50 µL elution buffer and incubate for 2 min at room temperature.
15. Collect RNA by spinning at 16,060*g* for 2 min.
16. Measure concentration of RNA at 260 nm (*see* **Note 24**).
17. Store at –80°C.

Table 1
Sequences of Primers and Conditions Used in Quantitative RT-PCR

Gene	MT	Mg conc.	Ex. time	Pro. length	Forward primer	Reverse primer
ALBP	87	3	11	207	TTGGTCACCATCCGGTCAGA	TTCCACCACCAGCTTGTCAC
Osteocalcin	90	4	11	198	TCTGACAAAGCCTTCATGTCC	AAATAGTGATACCGTAGATGCG
Col2a1	95	3	11	228	CCGTCATCGAGTACCGATCA	CAGGTCAGGTCAGCCATTCA
β-*Actin*	89	3	20	425	GGCCCAGAGCAAGAGAGGTATCC	ACGCACGATTTCCCTCTCAGC

MT, melting temperature (°C); Mg Conc., $MgCl_2$ concentration (m*M*); Ex. Time, extension time (s); Pro. Length, product length (bp).

Table 2
Components for Quantitative RT-PCR Mixture

$MgCl_2$ concentration (m*M*)	2	3	4	5
cDNA (µL)	5	5	5	5
Primers* (µL)	0.5	0.5	0.5	0.5
$MgCl_2$ (25 m*M*) (µL)	0.4	0.8	1.2	1.6
Master mixture (µL)	1	1	1	1
Distilled water (µL)	3.1	2.7	2.3	1.9

*5 pmol/µL each of sense and antisense primers.

3.4.1.2. Complementary DNA Synthesis (*see* **Note 25**)

1. Prepare the following components: A mix: add 0.1–1 µg RNA, 1 µL random hexamer (50 ng/mL), 1 µL dNTP (2.5 m*M* each), and diethylpyrocarbonate-treated water to make a total volume of 10 µL; B mix: combine 2 µL 10X RT buffer, 4 µL 25 m*M* $MgCl_2$, 2 µL 0.1 *M* dithiothreitol, and 1 µL RnaseOUT recombinant RNase inhibitor.
2. Incubate A at 65°C for 5 min and leave on ice for 1 min.
3. Mix A and B and leave at room temperature for 2 min.
4. Add 1 µL (50 U) SuperScript II RT and incubate at room temperature for 10 min.
5. Incubate at 42°C for 50 min and then at 70°C for 15 min.
6. Add 1 µL (2 U) ribonuclease H and incubate at 37°C for 20 min.
7. Add 80 µL dH_2O and store at –20°C.

3.4.1.3. Quantitative RT-PCR (*see* **Note 26**)

ALBP, *osteocalcin*, and *col2a1* are used for detection of adipocytes, osteoblasts, and chondrocytes, respectively. Primers for each gene as well as β-*actin* primers are shown in **Table 1**.

1. Prepare PCR reaction mixture as shown in **Table 2**.
2. Prepare standard curve control using a four times serial dilution of plasmid containing fragments of each gene (*see* **Note 27**).
3. Run PCR reaction as follows: preincubate at 95°C for 5 min; amplify for 45 cycles of 95°C for 15 s, 58°C for 5 s, and 72°C for various times (*see* **Table 1**) to detect fluorescence at the end of extension.
4. For the melting curve, run PCR at 95°C for 0 s, 65°C for 15 s, increase the temperature at a rate of 0.1°C/s and continuously check fluorescence until 95°C is reached.
5. For data analysis, check if single peak is obtained from each sample with the same melting temperature of the standard (**Table 1**).
6. Calculate relative number of genes using standard curve.
7. Determine relative expression level by normalization using β-*actin* expression level.

3.4.2. Histochemical Staining

Histochemical staining is carried out for the functional analysis of adipocytes, osteoblasts, and chondrocytes. Oil droplet deposition in adipocytes, calcium deposition by osteoblasts, and production of acidic proteoglycans by chondrocytes could be detected by Oil Red O, alizarin red, and alcian blue staining, respectively (**Fig. 1B**).

3.4.2.1. Oil Red O Staining

1. Aspirate off the medium and rinse the cells twice with PBS.
2. Fix with 10% formalin in PBS for 5 min.
3. Remove formalin and wash twice with distilled water.
4. Incubate with 60% isopropyl alcohol for 1 min and remove.
5. Incubate with Oil Red O solution for 10 min and remove.
6. Incubate with 60% isopropyl alcohol for 2 min to remove excess staining.
7. Wash several times with distilled water.
8. Store in 75% glycerol.

3.4.2.2. Alizarin Red Staining

1. Aspirate off the medium and rinse the cells twice with PBS.
2. Fix with 10% formalin in PBS for 5 min.
3. Remove formalin and wash twice with distilled water.
4. Incubate with alizarin red solution for 5 min.
5. Remove alizarin red solution and wash several times with distilled water.
6. Allow to dry.
7. To quantify, incubate alizarin red-stained samples with 4 *M* guanidine HCl at 37°C overnight.
8. Dissolve completely by pipetting and transfer 200 µL solution to 96-well flat plate.
9. Measure amount of alizarin red spectrophotometrically at 490 nm.

3.4.2.3. Alcian Blue Staining

1. Aspirate off the medium and rinse the cells twice with PBS.
2. Fix with 4% paraformaldehyde for 5 min.
3. Wash twice with distilled water.
4. Incubate cells with 1% alcian blue solution for 30 min.
5. Wash twice with 0.1 *N* HCl.
6. Allow to dry.

3.5. Optional Dissociation and Reaggregation of EB Outgrowths (see Note 28)

Identification and purification of progenitors of mesenchymal cells requires dissociation of EBs. However, dissociation of EBs after RA treatment drastically reduces subsequent mesengenesis. This may be because progenitors of mesenchymal cells are sensitive to environmental change caused by dissociation at this early time point. However, I found that dissociated cells 6 d after plating keep their potential to be mesenchymal cells as judged by RT-PCR. Here, a method for the dissociation of EBs and subsequent mesengenesis in reaggregates is shown. Typical results of mesengenesis using various reagents are shown in **Table 3**.

3.5.1. Osteogenesis and Chondrogenesis

1. Prepare EBs by hanging drop method and treat them with RA as in **Subheading 3.2.**
2. Plate EBs on gelatin-coated dish and culture for 6 d with osteogenesis medium.
3. Rinse plates twice with PBS and incubate with collagenase/dispase at 37°C for 20–30 min.
4. Collect cells in a universal and incubate plate with trypsin at 37°C for 5 min to dissociate remaining cells adhered to the plate.

Table 3
Conditions for Mesengenesis of Reaggregates

	Reagents used after dissociation			Initial culture
	Insulin/T_3	BMP-4/Dex/β-GP/AA	TGF-β_3/PTH/1% FBS	
ALBP	○	○	x	No BMP
	x	x	x	BMP
Osteocalcin	x	○	x	BMP
Col2a1	x	x	○	BMP

5. Add medium and dissociate cells by pipetting.
6. Centrifuge at 260*g* for 3 min and count cells.
7. Add medium to a final concentration of 10^6 cells/mL.
8. Aliquot 200 µL each in 96-well (V shape) and centrifuge at 260*g* for 5 min.
9. Allow to form aggregates at 37°C overnight.
10. Collect aggregates using a yellow tip and plate on gelatin-coated 96-well plate (flat bottom).
11. Culture in osteogenesis or chondrogenesis medium for 3 wk.
12. Change half of medium every 2 or 3 d.

3.5.2. Adipogenesis

1. For adipogenesis, culture EB outgrowth without BMP-4 for 6 d (*see* **Note 29**).
2. Make aggregates and plate as in **Subheading 3.5.1.**
3. Culture with adipogenic medium for 2–3 wk until adipocytes with oil droplets emerge (*see* **Note 30**).
4. Change half of medium every 2 or 3 d.

4. Notes

1. Excess NH_4OH in alizarin red results in precipitation of the dye.
2. Tissue culture-grade water should be used to prepare any materials in this subheading.
3. FBS and LIF are enough to maintain pluripotency of ES cells without feeders.
4. Mesengenesis has been tested using CGR8, EFC1, and E14Tg2a ES cell lines. Among them, CGR8 gives the best results for adipogenesis, osteogenesis, and chondrogenesis. Chondrogenesis from E14Tg2a is poor.
5. The number of cells reaches several million. Confluence is reached at around 5 million cells.
6. To minimize the presence of differentiated cells in ES cell culture, plate ES cells on nongelatin-coated dish and incubate for 30 min to allow differentiated cells to adhere to the plate. Recover ES cells by mild rinsing of the plate surface and seed them on a gelatin-coated dish.
7. ES medium without LIF is used throughout this subheading.
8. Short time exposure of trypsin and mild tapping helps to eliminate differentiated cells from ES cell culture as ES cells detach from the plate more easily than differentiated cells.
9. Because of surface tension, 10 mL PBS is not enough to cover the surface of a 90-mm bacteriological dish.
10. Approximately 100 drops can be made in each lid (**Fig. 2B**).
11. Make drops quickly and put plate in the incubator immediately after drop formation because the pH of the drops changes quickly.

12. 1 d is enough to collect EBs. In this case, expose EBs to RA for 4 d from d 1–5.
13. If differentiated cells are present in high numbers in ES cell culture, then several EBs will be formed in each drop, which is not ideal for following mesengenesis. *See* **Notes 6** and **8** to minimize the number of differentiated cells in ES cell culture.
14. Try to work in a dark place when treating with RA.
15. The effect of RA treatment can be confirmed by suppression of cardiomyocyte formation in EB outgrowths. Generally, 80% of EB outgrowths form cardiomyocytes and start beating within 7 d after plating. On RA treatment, this ratio is drastically reduced to less than 10%.
16. It is recommended to use plastic pipets to collect EBs as they have a larger diameter tip than glass pipets.
17. BMP-4 is essential for ALP activities and Dex/β-GP/AA is required for calcium deposition. However, in the case of E14Tg2a, Dex/β-GP/AA is also essential for ALP activities.
18. Although PTH generally induces chondrogenesis, TGF-β_3 is the determining factor in this protocol as judged by expression of *Col2a1*.
19. Adipocytes with oil droplets start to appear around 10 d after plating.
20. Transient markers of chondrogenesis are detectable in the osteoblast condition at around d 6. Osteogenesis starts after this period. Markers for osteoblasts are readily detectable from d 9, and calcium deposition occurs from d 12.
21. Chondrogenesis begins after d 15 as judged by expression of *Col2a1*.
22. All components of this subheading are provided in each kit.
23. This protocol is based on the Absolutely RNA RT-PCR miniprep kit.
24. Use the following equation for calculation: ng/μL = $abs_{260nm} \times 40 \times$ dilution factor.
25. This protocol is based on the SuperScript first-strand synthesis system for RT-PCR.
26. This protocol is based on the LightCycler-fast start DNA master SYBR green I and LightCycler software v3.5. The PCR reaction is carried out in a LightCycler.
27. Plasmid containing a fragment of a gene is obtained by cloning of PCR product using TOPO TA cloning kit. Sequence of the insert is confirmed using M13 forward and reverse primers.
28. ES medium without LIF is used throughout this subheading unless otherwise mentioned.
29. Exposure of EB outgrowths with BMP-4 for the first 6 d drastically reduces subsequent adipogenesis (**Table 3**).
30. Under these conditions, adipocytes will also develop in osteogenesis medium (**Table 3**).

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11

Analysis of Embryonic Stem Cell-Derived Osteogenic Cultures

Nicole L. Woll and Sarah K. Bronson

Summary

The process of bone formation can be approximated in vitro in the form of a mineralized nodule. Osteoprogenitors and mesenchymal stem cells, the immediate precursors to the osteoprogenitor, when placed into culture proliferate and differentiate into osteoblasts. These osteoblasts secrete and mineralize a matrix during a period of 3–4 wk. The differentiation potential of embryonic stem cells (ESCs) suggests that ESCs should also have the ability to form bone nodules in vitro. ESCs were allowed to form embryoid bodies, which were disrupted and plated at concentrations as low as 25 cells/cm^2. By 7 d postplating, a significant percentage of the colonies were morphologically characteristic of other types of osteogenic cultures. By 3 wk in culture, these colonies go on to form layered nodules. In a typical experiment, approx 60% of the colonies contain mineralized nodules, as revealed by staining of fixed cultures. Quantitative reverse transcriptase polymerase chain reaction analysis for genes characteristic of the osteoblast lineage has been used to confirm the presence of mature osteoblasts. Differentiation of ESCs into the osteoblast lineage will be a valuable tool for addressing pertinent questions about the proliferation, differentiation, survival, and intercellular communication between cells of the bone lineage in vitro.

Key Words: Bone; embryonic stem cells; osteoblasts; Osteocalcin; Runx2; stem cell differentiation; Type I collagen.

1. Introduction

The bone tissue of vertebrate skeletons is comprised of several cell types. Of these, the osteoblast secretes and mineralizes the bone matrix during both intramembranous and endochondral bone development, as well as during bone repair and remodeling in adults *(1–3)*. Mesenchymal stem cells or marrow stromal cells (MSCs) are the immediate progenitors to the osteoblast lineage. Differentiation proceeds through characteristic stages of commitment, proliferation, matrix secretion, and mineral deposition and results in a resting osteoblast in the periosteum, an osteocyte embedded within the matrix, or apoptosis of the terminally differentiated cell. The process of bone formation can be approximated in vitro in the form of a mineralized nodule. Many laboratories

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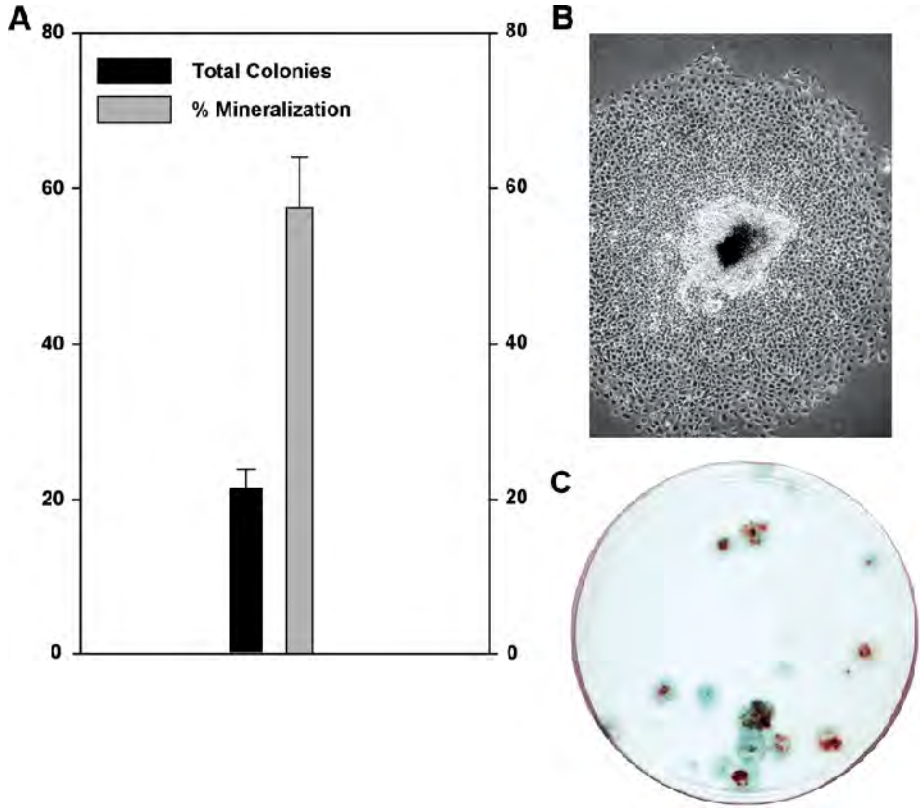

Fig. 1. (**A**) Total colony forming units and percentage of colonies with mineralization after 21 d in culture. (**B**) Osteogenic cultures stained at d 21 using a silver nitrate/methyl green staining technique. (**C**) Phase contrast image (×40) of embryonic stem cell-derived osteogenic colony 7 d postplating. (Please *see* the companion CD for the color versions of this figure.)

have differentiated MSCs or osteoprogenitors from fetal or neonatal calvaria or from bone marrow *(4,5)*. Individual cells, when placed in culture, will establish osteogenic colonies with characteristic morphology, gene expression, and mineralized bone matrix.

Embryonic stem cells (ESCs) have the potential to differentiate into nearly all cell lineages *(6,7)*. The differentiation potential of murine ESCs can be preserved in the presence of leukemia inhibitory factor (LIF) and fetal bovine serum (FBS) *(7)*. ESC colonies can be flushed from a feeder layer; in the absence of LIF and with forced suspension, embryoid bodies (EBs) will form, and differentiation is initiated. We have utilized this method to generate MSC-like progenitors for the initiation of clonal osteogenic colonies capable of forming mineralized nodules in vitro. After trypsinization of d 2 EBs, single cells are plated at varying concentrations in osteogenic medium containing ascorbic acid and glycerophosphate β-(GP), additives documented to improve matrix structure and mineral deposition *(4,5)*. Typically, 21 d after plating 2×10^3 cells/100-mm dish, we see 20–40 colony-forming units (CFUs) with approx 60% of these colonies layered and mineralized, yielding a CFU-osteogenic frequency of approx 1 in 100 EB-derived cells (*see* **Fig. 1**).

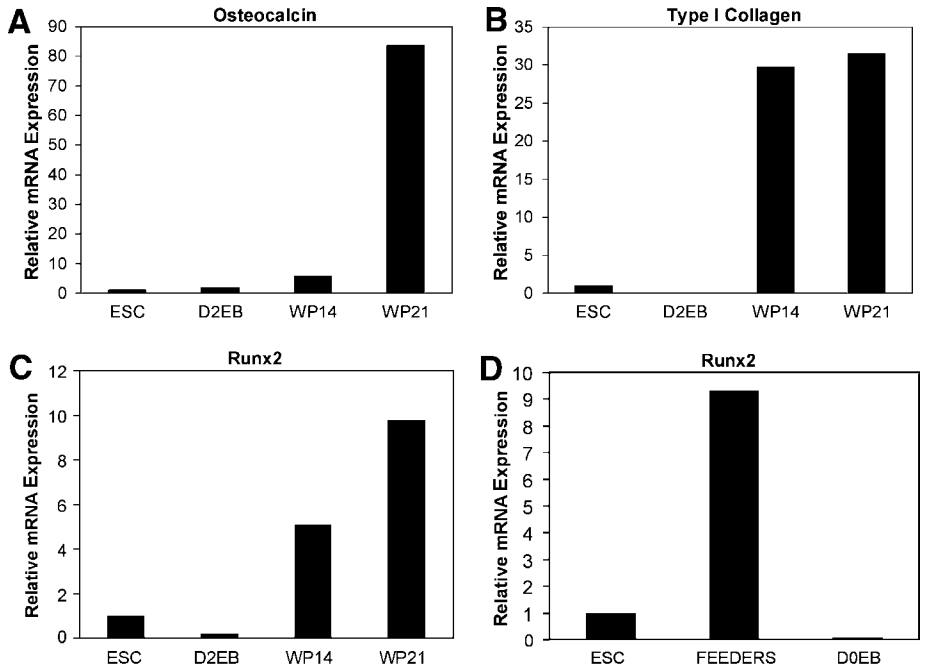

Fig. 2. Real-time polymerase chain reaction (PCR) analysis of osteoblast differentiation markers (**A**) Osteocalcin, (**B**) Type I collagen, and (**C**) Runx2 for mineralized nodule formation. Real-time PCR analysis on Runx2 (**D**) gene expression in mitotically inactivated fibroblast feeders in comparison with ESCs and d 0 embryoid bodies. Assays were designed to detect only RNA. Each assay was run in duplicate at two different template concentrations. Relative mRNA expression is normalized to ribosomal protein L7 (Rpl7) and displayed relative to ESC. ESC, embryonic stem cells; D0EB, d 0 embryoid body; D2EB, d 2 embryoid body; WP14, whole plate d 14; WP21, whole plate d 21.

Although the cellular morphology and mineralization of the nodules suggests that these colonies are osteogenic, we have further defined the differentiation process in these cultures using quantitative real-time polymerase chain reaction (QRT-PCR) analysis of cDNA from entire plates and individual colonies (*see* **Figs. 2** and **3**). Type I collagen is a principle gene product of the osteoblast and comprises approx 90% of the protein content in the bone matrix. Osteocalcin is the most abundant noncollagenous protein in the matrix, with secretion initiating just after the onset of mineralization, and is the most specific marker of the mature osteoblast. Runx2, also referred to as cure binding factor α1, is an osteoblast-specific transcription factor that regulates genes important for the formation and mineralization of the bone matrix. Type I collagen, osteocalcin, and Runx2 are transcriptionally upregulated in the osteogenic culture as differentiation progresses ***(8)***, and we observed similar upregulation of these genes in individual colonies.

Our method of initiating osteogenesis from ESC cultures is the only described method capable of observing and manipulating the commitment stage of mesengenesis. Other methods involve the deposition of entire EBs ***(9,10)*** or very high densities of cells ***(11)***

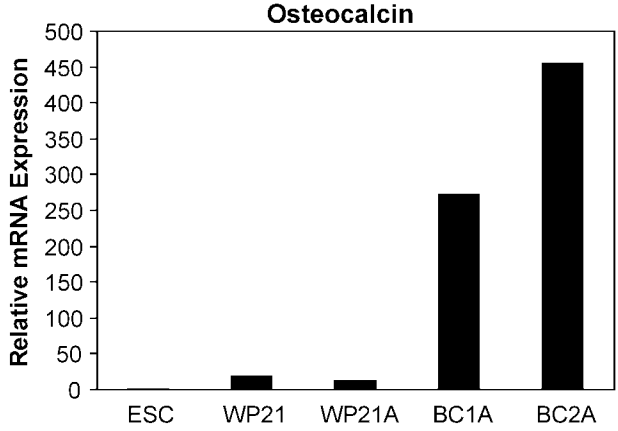

Fig. 3. Real-time polymerase chain reaction analysis of osteocalcin expression in individual bone colonies at various stages of mineralized nodule formation. ESC, embryonic stem cell; WP21, whole plate d 21; WP21A, whole plate d 21 amplified; BC1A, bone colony 1 amplified; BC2A, bone colony 2 amplified.

and are limited to measures of differentiation capacity. Further, we find relatively high levels of mesenchymal progenitors, approx 1/100 in the absence of exogenous steroidogenic hormones, peptide growth factors, or cytokines.

2. Materials

2.1. ESCs and EB Media

1. ESC medium (D15-LIF): 500 mL Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA; cat. no. 11965-092), 75 mL ES cell-qualified FBS (Atlanta Biologicals, Norcross, GA; cat. no. S11550), 6 mL 100X Glutamax (Invitrogen, cat. no. 35050-061), 4 µL mercaptoethanol β-(ME) (Sigma, St. Louis, MO; cat. no. M-7522), approx 1000 U/mL LIF (Chemicon, Temecula, CA; cat. no. ESG1106); and 6 mL 100X sodium pyruvate (Invitrogen, cat. no. 11360-070).
2. Gelatin (0.1%): 0.1 g gelatin and 100 mL distilled deionized water (ddH_2O). Autoclave gelatin solution prior to use and store at room temperature.
3. Solution B: 4 g NaCl, 0.2 g KCl, 0.175 g $NaHCO_3$, 0.5 g glucose, and 0.1 g ethylenediaminetetraacetic acid (free acid). Combine with 475 mL ddH_2O. Stir overnight to completely dissolve; add NaOH to pH 7.0. Bring to 500 mL, filter thru 0.22-µm filter, and store at 4°C.
4. Trypsin solution: 80 mL 0.25% trypsin (Invitrogen, cat. no. 15050-065) and 420 mL solution B. Aliquot for single use and store at –20°C.
5. EB medium (D15): 500 mL DMEM (Invitrogen, cat. no. 11965-092); 75 mL ESC-qualified FBS (Atlanta Biologicals, cat. no. S11550); 6 mL 100X Glutamax (Invitrogen, cat. no. 35050-061); 4 µL β-ME (Sigma, cat. no. M-7522); and 6 mL 100X sodium pyruvate (Invitrogen, cat. no. 11360-070).
6. PBS (1X): 50 mL 10X PBS (Invitrogen, cat. no. 70011-044) and 450 mL ddH_2O. Filter through a 500-mL 0.22-µm filter unit (Fisher Scientific, Pittsburgh, PA; cat. no. 097615).

2.2. Osteogenic Culture

1. 1000X L-ascorbic acid 2-phosphate (sesquimagnesium salt): 0.5 g L-ascorbic acid 2-phosphate (Sigma, cat. no. A8960) and 10 mL ddH_2O. Filter through a 0.22-µm filter. Stable for 1 mo at 4°C.
2. Osteogenic medium (I10, aa): 500 mL Iscove's modified Dulbecco's medium (Invitrogen, cat. no. 12240-053); 50 mL FBS (Invitrogen, cat. no. 10437-028); 6 mL 100X Glutamax (Invitrogen, cat. no. 35050-061); 4 µL β-ME (Sigma, cat. no. M-7522); and 0.6 mL 1000X L-ascorbic acid 2-phosphate stock.
3. 100X β-GP: 29.7 g β-GP (Sigma, cat. no. G9891). Bring to 100 mL with distilled deionized water. Aliquot and store at –20°C.
4. 7-µm cell strainer (Fisher Scientific, cat. no. 087712).

2.3. Silver Nitrate/Methyl Green Staining

1. Silver nitrate: 2.5 g silver nitrate (Sigma, cat. no. S0139) and 100 mL ddH_2O.
2. Methyl green (Vector Labs, Burlingame, CA; cat. no. H3402).

2.4. Gene Expression in Osteogenic Cultures

1. ABI PRISM Sequence Detection System (Applied Biosystems, Foster City, CA; model no. 7700).
2. QuantiTect SYBR Green PCR master mix (Qiagen, Valencia, CA; cat. no. 204143).
3. TriReagent (Molecular Research Center, Inc., Cincinnati, OH; cat. no. TR 118).
4. RNeasy minikit (Qiagen, cat. no. 74104). β-ME must be added to buffer RLT before use: add 10 µL β-ME per 1 mL buffer RLT. This solution is stable for 1 mo at room temperature.
5. Message Amp aRNA kit (Ambion, Austin, TX; cat. no. 1750).
6. RETROscript kit (Ambion, cat. no. 1710).

3. Methods

3.1. Thawing and Maintaining ESCs

1. Thaw ESCs quickly from vials maintained in liquid nitrogen.
2. Immediately place in 10 mL ESC medium.
3. Collect the cell pellet by centrifugation at 200*g* for 10 min.
4. Remove the medium and resuspend the cell pellet in fresh ESC medium.
5. Culture ESCs on a mitotically inactivated fibroblast feeder layer plated on 0.1% gelatin-coated 100-mm tissue culture plates.
6. Grow ESCs to subconfluence, trypsinize, and split 1:3 to 1:10 to a nearly single-cell suspension every 2–3 d (*see* **Note 1**).

3.2. EB Formation

1. Wash ESCs twice with 3 mL 1X PBS.
2. Trypsinize cells with 2 mL trypsin and immediately incubate 3–5 min at 37°C.
3. Tap plates to release the colonies from attachment to the bottom of the plate.
4. When cells have reached a nearly single-cell suspension, add 2 mL D15-LIF and pipet to mix.
5. Bring the volume to 10 mL and pipet to mix.
6. Plate cells densely (1:1 to 1:3) back onto the original plate and incubate overnight at 37°C.
7. The next day, wash the ESCs off the plate with 8 mL D15 medium and pipet onto four Petri dishes. This step is repeated twice. Feeders should remain on the plate.

8. Bring the volume of the Petri dish to 8 mL.
9. Incubate the EBs for 2 d at 37°C without additional feeding.

3.3. Establish Osteogenic Culture

1. Pipet EBs off the dish and through a 70-µm cell strainer onto the original Petri dish. Discard the flow through.
2. Turn the cell strainer upside-down and pipet 10 mL I10, aa medium over the strainer into a 50-mL conical tube.
3. Allow the EBs to settle in the conical tube for about 3–5 min and then aspirate the medium, taking care not to aspirate the loose EB pellet.
4. Wash EBs twice with 3 mL 1X PBS; after the second wash, pellet EBs at 200*g* for 3 min.
5. Add 2 mL trypsin and incubate in 37°C water bath with shaking 3–5 min until trypsin is cloudy and no EBs are visible.
6. Add 2 mL I10, aa medium and pipet to mix.
7. Bring cells through an 18-gage needle three times.
8. Pipet cells through a 70-µm cell strainer into a 50-mL conical tube.
9. Bring volume to 20 mL with I10, aa medium and pipet to mix.
10. Take an aliquot out to count and pellet the remaining cells down at 200*g* for 10 min.
11. Calculate amount of I10, aa medium needed to resuspend the cell pellet and plate at the desired concentrations in 100-mm tissue culture plates (*see* **Note 2**).
12. Incubate at 37°C in a humidified 5% CO_2 chamber. Refresh with osteogenic medium every 2–3 d.
13. Add 2 n*M* β-GP to the medium at d 7 to aid mineral deposition (*see* **Note 3**).

3.4. Fixation and Staining

3.4.1. Fixation

1. Wash plates with 3 mL cold 1X PBS.
2. Add 5 mL cold 10% formalin and incubate for 2 h at room temperature or at 4°C for an extended period of time.

3.4.2. Silver Nitrate/Methyl Green Staining

1. Rinse plates with distilled deionized water and aspirate.
2. Add 7 mL 2.5% silver nitrate (*see* **Note 4**).
3. Incubate 1 h under ultraviolet light.
4. Wash plates twice with distilled deionized water.
5. Add 5 mL methyl green and leave on plates 30 s.
6. Rinse plates three times with distilled deionized water (*see* **Note 5**).
7. Air-dry plates. **Figure 1A** shows total CFUs and percentage of colonies with mineralization after 21 d in culture. Typically, 60% of the total CFUs are mineralized. **Figure 1B** shows a phase contrast image (×40) of an ESC-derived osteoblast colony 7 d postplating. By 7 d postplating, we are able to identify colonies with morphology characteristic of other types of osteogenic cultures. The outer portion of the colony is still proliferating; the inner portion has begun to layer and mineralize. **Figure 1C** shows an osteogenic culture plate stained at d 21 using a silver nitrate/methyl green staining technique.

3.5. Characterization of Gene Expression in Osteogenic Cultures

To confirm that the osteogenic cultures were expressing genes characteristic of the osteoblast lineage, QRT-PCR was conducted. QRT-PCR was performed using an ABI

PRISM 7700 Sequence Detection System and the QuantiTect SYBR Green PCR master mix. The SYBR Green PCR amplifications, using primers for Type I collagen, Osteocalcin, and Runx2, confirmed that genes characteristic of the osteoblast lineage were expressed in the cultures (*see* **Fig. 2A–C**). **Figure 3** shows osteocalcin gene expression in individual bone colonies.

3.5.1. RNA Isolation From Osteogenic Culture

RNA is collected using TriReagent according to the manufacturer's protocol.

1. Wash plate once with 1X PBS.
2. Add 2 mL TriReagent to each 100-mm osteogenic culture plate.
3. Collect the solubilized material and divide into two microfuge tubes.
4. Incubate at room temperature for 5 min.
5. Add 200 µL chloroform and incubate at room temperature for 15 min.
6. Centrifuge tubes 15 min at 19,000*g* at 8°C.
7. Remove aqueous phase, add to 500 µL isopropanol, and incubate at room temperature for 10 min.
8. Centrifuge 15 min at 19,000*g* at 8°C.
9. Wash RNA pellet with 1 mL 70% ethanol and centrifuge for 10 min at 19,000*g*.
10. Air-dry pellet, resuspend in 50 µL 1 m*M* ethylenediaminetetraacetic acid, and incubate at 68°C for 10 min. Pipet to resuspend.
11. Store at −80°C.

3.5.2. RNA Isolation From Individual Colonies

RNA isolation from individual colonies was performed using the Qiagen RNeasy minikit with minor modifications (*see* **Note 6**).

1. Aspirate medium from a 100-mm plate.
2. Wash plate with 1X PBS and aspirate off PBS.
3. Pipet 350 µL RLT/β-ME buffer into RNase-free microfuge tubes (one tube/colony).
4. Pick colonies from plate and place in RLT/β-ME buffer in microfuge tube by mouth pipetting a small volume of RLT/β-ME buffer onto colony using a flamed-pulled glass capillary, dislodging a colony and pipetting it into the buffer.
5. Vortex tubes until colonies are broken up.
6. Pipet lysate directly onto Qiashredder column sitting in a 2-mL collection tube (both provided with kit).
7. Discard the column, add 1 vol 70% ethanol to the homogenized lysate, and mix well by pipetting (approx 300 µL 70% ethanol).
8. Transfer the sample (up to 700 µL) to an RNeasy minispin column sitting in a 2-mL collection tube and centrifuge for 15 s at 19,000*g*.
9. Discard the flow through and add 700 µL RWI buffer onto the RNeasy column.
10. Centrifuge for 15 s at 19,000*g*.
11. Discard the flow through and the collection tube.
12. Transfer the RNeasy column into a new 2-mL collection tube.
13. Add 500 µL RPE buffer onto the RNeasy column and centrifuge for 15 s at 19,000*g*.
14. Discard the flow through and reuse the collection tube for the next step.
15. Add 500 µL RPE buffer onto the RNeasy column and centrifuge for 2 min at 19,000*g* to dry the RNeasy membrane.

16. Discard the flow through and spin an additional minute.
17. Transfer RNeasy column into a 1.5-mL collection tube and add 30 µL RNase-free water directly onto the RNeasy membrane.
18. Centrifuge for 1 min at 19,000*g* to elute RNA. Discard column and store RNA at −80°C.

3.5.3. Amplification of RNA From Individual Colonies

RNA isolation from individual colonies does not yield a high enough concentration of RNA to be used in SYBR Green QRT-PCR. Therefore, the RNA from individual colonies is amplified using the Message Amp aRNA kit with minor modifications (*see* **Note 7**). Prior to amplification, the RNA purity and integrity are checked using an Agilent 2100 Bioanalyzer.

3.5.3.1. First-Strand cDNA Synthesis

1. Place up to 5 µg total RNA into an RNase-free microfuge tube.
2. Add 1 µL T7 Oligo (dT) primer.
3. Add nuclease-free water to a final volume of 12 µL.
4. Incubate 10 min at 70°C in a thermocycler without heated lid.
5. Centrifuge briefly and place mixture on ice.
6. Prepare RT master mix (1X): 2 µL 10X first-strand buffer, 1 µL ribonuclease inhibitor, and 4 µL dNTP mix.
7. Mix gently, centrifuge, and place on ice.
8. Transfer 7 µL RT master mix to each RNA sample.
9. Mix gently by pipetting and place tubes in a 42°C incubator.
10. Quickly add 1 µL RT to each sample.
11. Incubate samples for 2 h at 42°C, centrifuge briefly, and place tubes on ice.

3.5.3.2. Second-Strand Synthesis

1. Prepare a master mix of second-strand cDNA synthesis reagents (1X): 63 µL nuclease-free water, 10 µL 10X second-strand buffer, 4 µL dNTP mix, 2 µL DNA polymerase, and 1 µL RNase H.
2. Place 80 µL second-strand cDNA synthesis reagents into each 20-µL first-strand cDNA synthesis reaction.
3. Gently pipet up and down and centrifuge briefly.
4. Incubate 2 h at 16°C in a thermocycler without heated lid.
5. Continue to cDNA purification or place tubes at −20°C.

3.5.3.3. cDNA Purification

1. Preheat nuclease-free water to 50°C for at least 10 min.
2. Equilibrate one cDNA filter cartridge per cDNA sample: pipet 50 µL cDNA binding buffer onto the filter in the cDNA filter cartridge seated in a 2-mL wash tube and incubate at room temperature for 5 min.
3. Add 250 µL cDNA binding buffer to each cDNA sample and mix thoroughly.
4. Apply mixture to an equilibrated cDNA filter cartridge: pipet cDNA sample/cDNA binding buffer onto the center of the filter cartridge and centrifuge 1 min at 9700*g*. Discard flow through.
5. Wash the cDNA filter cartridge with 650 µL cDNA wash buffer: apply 650 µL cDNA wash buffer to each filter and centrifuge 1 min at 9700*g*. Discard flow through, spin an additional minute, and transfer cDNA filter cartridge to a cDNA elution tube.

6. Elute cDNA twice with 10 µL preheated nuclease-free water: apply 10 µL nuclease-free water to the center of the filter and leave at room temperature for 2 min, then centrifuge 1.5 min at 9700*g*. Repeat elution; discard cDNA filter cartridge.
7. Check to make sure volume is about 16 µL; if not, then add nuclease-free water. Store at –20°C.

3.5.3.4. In Vitro Transcription to Synthesize aRNA

1. Assemble the 40-µL transcription reaction at room temperature in the order shown (1X): 4 µL T7 ATP solution; 4 µL T7 CTP solution; 4 µL T7 GTP solution; 4 µL T7 UTP solution; 4 µL 10X reaction buffer; and 4 µL T7 enzyme mix.
2. Gently pipet and centrifuge briefly.
3. Add 24 µL reaction to 16 µL double-stranded cDNA from **Subheading 3.5.3.3.**, **step 7**.
4. For cDNA purification, incubate for 6–14 h at 37°C and add 60 µL elution solution to each aRNA sample. Mix thoroughly.

3.5.3.5. aRNA Purification

1. Preheat elution solution to 50–60°C for 10 min.
2. Equilibrate aRNA filter cartridge for each sample: place an aRNA filter cartridge in an aRNA collection tube and add 100 µL aRNA binding buffer. Incubate at room temperature for 5 min.
3. Add 350 µL aRNA binding buffer to each aRNA sample and mix thoroughly.
4. Add 250 µL ACS grade 100% ethanol to each aRNA sample and mix thoroughly.
5. Apply mixture to equilibrated aRNA filter cartridge: pipet onto the center of aRNA filter cartridge and centrifuge for 1 min at 9700*g*. Discard flow through.
6. Wash the aRNA filter cartridge with 650 µL aRNA wash buffer: pipet onto the center of cartridge and centrifuge for 1 min at 9700*g*. Discard flow through and spin an additional minute. Transfer filter cartridge to a fresh aRNA collection tube.
7. Elute aRNA twice with 50 µL preheated elution solution: add 50 µL elution solution to the center of cartridge and return the elution solution to 50°C. Leave at room temperature for 2 min and centrifuge 1.5 min at 9700*g*.
8. Repeat **step 7** and discard aRNA filter cartridge. The final volume should be 100 µL. Store at –80°C.

3.5.4. SYBR Green QRT-PCR

3.5.4.1. RT Reaction

Reverse transcribe 2 µg RNA with the RETROscript kit using an Oligo (dT) primer.

1. Prepare RT reaction mix (1X): 2 µg RNA, 2 µL oligo dT, and distilled deionized water to bring volume to 12 µL.
2. Heat at 78°C for 3 min.
3. Add 2 µL RT buffer, 4 µL dNTPs, 1 µL RNase inhibitor, and 1 µL RT.
4. Conduct the RT reaction with heat denaturation of the RNA.
5. Incubate the reaction at 42–44°C for 1 h and then at 92°C for 10 min to inactivate the RT.

3.5.4.2. Polymerase Chain Reaction

For the PCR, set up PCR reaction mix (1X) of 0.4 µ*M* of each of the appropriate primers (*see* **Note 8**), 5 µL diluted cDNA, 16 µL H_2O, and 25 µL SYBR Green reaction mix. *See* PCR Conditions in **Fig. 4** (*see* **Note 9**).

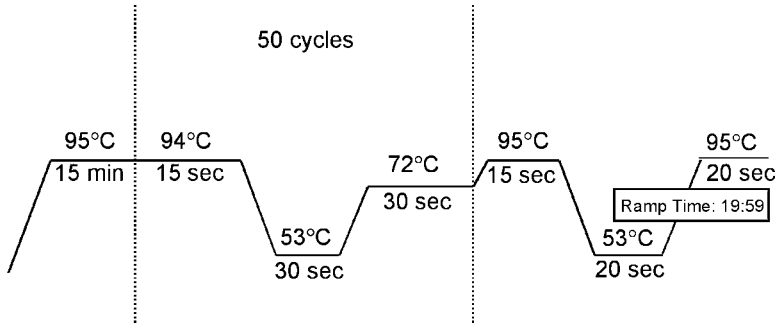

Fig. 4. Schematic representation of polymerase chain reaction (PCR) conditions for SYBR green quantitative real-time PCR. Annealing temperature of 53°C can vary depending on melting temperature of primers.

Table 1
Primer Sequences for QRT-PCR

Gene	Forward primer 5′ → 3′	Reverse primer 5′ → 3′
Rpl7	CCCTGAAGACACTTCGAAAGG	GCTTTCCTTGCCATCCTAGC
Osteocalcin	CTACCTTGGAGCCTCAGTCC	TTAGGGCAGCACAGGTCCTA
Type I Collagen	CCGGAAGAATACGTATCACC	ACCAGGAGGACCAGGAAGTC
Runx2	GGAACCAAGAAGGCACAGACA	AATGCGCCCTAAATCACTGAG

4. Notes

1. ESC cultures are maintained in humidified incubators at 37°C and 5% CO_2. ESCs for osteogenic cultures are initiated within two passages of the thaw. No antibiotics are used in any of the cultures. These experiments were performed with the HM1 cell line, a rederivation of E14TG2a *(12)*, although several other ESC lines perform similarly *(13)*.
2. We typically plate 2×10^3 cells per plate for counting at 21 d postplating.
3. Adding β-GP to the osteogenic cultures too early can inhibit proliferation.
4. Make silver nitrate fresh as needed.
5. If staining more than one plate, then add methyl green and rinse once for each plate, then continue rinsing twice.
6. All steps performed in this protocol should be done at 20–25°C. All solutions used in this protocol are supplied with Qiagen RNeasy minikit with the exception of β-ME and 70% ethanol.
7. All solutions used in this protocol are supplied with Message Amp aRNA kit with the exception of 100% ethanol.
8. The primers were designed to detect only RNA by having at least one primer span an exon/exon boundary in the messenger RNA sequence, so detection of the amplicon could only occur when the intron was spliced out. Primers were designed for an amplicon of 50–150 bp (*see* **Table 1**).
9. Serial dilutions of ESC cDNA were used to generate standard curves (log of dilution vs C_t value). Duplicate dilutions of 1:8 and 1:32 were analyzed. The data were normalized to the internal control Rpl7 and plotted relative to the expression of the gene of interest in the wild-type ESC (*see* **Note 10**).

10. We have since changed our reference for QRT-PCR from ESCs to d 0 EB to more accurately reflect the differentiation process. The ESC cultures contain fibroblast feeders that contribute to the gene expression of the cultures, whereas d 0 EBs are ESC cultures that have been flushed from the feeder layer prior to trypsinization. (*see* **Fig. 2D**).

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12

Generation of Chondrocytes From Embryonic Stem Cells

Jaspal Singh Khillan

Summary

Pluripotent embryonic stem (ES) cells have complete potential for all the primary germ layers, such as ectoderm, mesoderm, and endoderm. However, the cellular and molecular mechanisms that control their lineage-restricted differentiation are not understood. Although embryoid bodies, which are formed because of the spontaneous differentiation of ES cells, have been used to study the differentiation into different cell types, including neurons, chondrocytes, insulin-producing cells, bone-forming cells, hematopoietic cells, and so on, this system has limitations for investigating the upstream events that lead to commitment of cells that occur during the inaccessible period of development. Recent developments in human ES cells have offered a challenge to develop strategies for understanding the basic mechanisms that play a key role in differentiation of stem cell into specific cell types for their applications in regenerative medicine and cell-based therapies. A micromass culture system was developed to induce the differentiation of ES cells into chondrocytes, the cartilage-producing cells, as a model to investigate the upstream events of stem cell differentiation. ES cells were co-cultured with limb bud progenitor cells. A high percentage of differentiated cells exhibit typical morphological characteristics of chondrocytes and express cartilage matrix genes such as collagen type II and proteoglycans, suggesting that signals from the progenitor cells are sufficient to induce ES cells into the chondrogenic lineage. Degeneration of cartilage in the joints is associated with osteoarthritis, which affects the quality of life of human patients. Therefore, the quantitative production of chondrocytes can be a powerful resource to alleviate the suffering of those patients.

Key Words: Alternate splicing of collagen type II gene; cartilage; chondrocytes; collagen type II; differentiation; embryonic stem cells; limb bud progenitor cells; Oct4 transcription factor; proteoglycans.

1. Introduction

Pluripotent embryonic stem (ES) cells derived from the inner cell mass (ICM) of the blastocyst have the complete potential to form all the primary germ layers, such as ectoderm, mesoderm, and endoderm *(1,2)*. The cellular and molecular mechanisms

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that control their differentiation into specific lineage, however, have not been elucidated. In the presence of a cytokine leukemia inhibitory factor (LIF), ES cells can be maintained indefinitely without differentiation *(3)*. After microinjection into blastocysts, these cells integrate and populate all the lineages, including germline tissues such as ovary and testis *(4)*. These observations have led to the assumption that ES cells undergo the same series of developmental changes, such as differentiation into various cell lineages and tissue development as normal ICM. Despite this potential, only limited information is available about the mechanisms responsible for commitment of ES cells to specific cell types.

In the absence of LIF, ES cells differentiate spontaneously into embryoid bodies (EBs) *(5)*, which contain many types of cells, including neurons, chondrocytes, insulin-producing cells, bone-forming cells, and hematopoietic cells *(6)*. Because EBs contain cells that are committed to different lineages, they cannot be used for studies of cell fate determination. Recent developments in human ES cells have offered a challenge to develop strategies for understanding the basic mechanisms that play a key role in lineage-restricted differentiation to specific cell types for their applications in regenerative medicine and cell-based therapies.

This chapter describes a micromass culture system for induced differentiation of ES cells into chondrocytes, the cartilage-producing cells. This may be a useful model system for investigation of the upstream events of stem cell differentiation into chondrocytes. Injury or degeneration of cartilage in the joints is associated with osteoarthritis, which affects the quality of life of over 20 million people in the United States. The quantitative production of chondrocytes can be a powerful resource to alleviate the suffering of those patients.

ES cells were co-cultured with mesenchymal progenitor cells isolated from limb buds of developing embryos to induce their differentiation into chondrocytes. The differentiated cells exhibit typical morphological characteristics of chondrocytes and express cartilage matrix genes such as collagen type II and proteoglycans. No additional growth factors are required, suggesting that signals from the progenitor cells are sufficient to induce ES cells into the chondrogenic lineage. Almost 60% of ES cells differentiate into mature chondrocytes. The micromass culture described here can therefore be an excellent model to study the mechanisms of linage-restricted differentiation of stem cells and for quantitative production of chondrocytes.

2. Materials

2.1. Tissue Culture Media and Solutions

1. Primary fibroblast (PF) culture medium: 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (pen-strep) (Gibco-BRL, Gaithersburg, MD; cat. no. 15140-163) in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, cat. no. 12430-054).
2. ES cell medium: mix 90 mL FBS, 6 mL 10 m*M* nonessential amino acids (Gibco-BRL, cat. no. 11140-050), 6 mL 100X pen-strep, 1 mL β-mercaptoethanol (Gibco-BRL, cat. no. 21985-023), and 60 µL LIF (Chemicon International Inc., Temecula, CA; cat. no. 3275SB), which provides 1000 IU/mL and 500 mL DMEM. The medium should be stored at 4°C protected from light and should be used within 3–4 wk.

3. 0.1% gelatin solution: dissolve 1.0 g gelatin (Sigma, St. Louis, MO; cat. no. 6650) in 1 L deionized water. The autoclaved gelatin solution can be stored indefinitely in 50- to 100-mL aliquots.
4. Mitomycin C solution: prepare a stock solution of mitomycin C 0.5–1.0 mg/mL (Sigma, cat. no. M5030) in 1X PBS or sterilized water. The solution can be stored at 4°C protected from light for up to 1 wk.
5. Enhanced green fluorescence protein (EGFP) gene construct with neomycin phosphotransferase (neo) gene is purchased from Clonetech Inc. (Palo Alto, CA).
6. Alcian blue solution: mix 20 mg alcian blue powder (Sigma, cat. no. A3157) in 80% ethanol and 20% glacial acetic acid *(7)*. The solution is filtered to remove dye particles.
7. Petri dishes: Nunc four-well Petri dishes (Fisher Scientific, Suwanee, GA).

2.2. Equipment

1. Laminar flow tissue culture hood, microfuge centrifuge, and CO_2 incubator for tissue culture.
2. Stereomicroscope with ultraviolet attachment.
3. Surgical instruments such as two pairs of forceps and a pair of scissors for the collection of embryos.

2.3. Polymerase Chain Reaction Primers

1. Oct4: 5′-GGC GTT CTC TTT GGA AAG GTG TTC-3′; 5′-CTC GAA CCA CAT CCT TCT CT 3′.
2. Collagen II: 5′-GTG AGC CAT GAT CCG C-3′; 5′-GAC CAG GAT TTC CAG G-3′ *(8)*.
3. Neomycin: 5′-AGG ATC TCC TGT CAT CTC ACC TTG CTC CTG-3′; 5′- AAG AAC TCG TCA AGA AGG CGA TAG AAG GCG-3′.
4. Hypoxanthine phosphoribosyltransferase: 5′-GTA ATG ATC AGT CAA CGG GGG AC-3′; 5′-CCA GCA AGC TTG CAA CCT TAA CCA-3′.

3. Methods

3.1. Preparation and Culture of ES Cells

ES cells are cultured in ES medium over a monolayer of mitotically inactive fibroblasts (*see* **Note 1**) in a humidified incubator with 6% CO_2 at 37°C following methods described previously *(3)*. **Figure 1** shows typical colonies of ES cells after 48 h. The distinct individual colonies of ES cells can be seen scattered throughout the plate. The ES medium supplemented with 1000 IU/mL LIF prevents the differentiation of ES cells (*see* **Note 2**).

1. Gelatinize a 60-mm plate with 0.1% gelatin for at least 2 h.
2. Transfer 1×10^6 mitomycin C-treated or irradiated primary fibroblast cells (*see* **Note 1**).
3. Allow cells to settle overnight and change to ES medium the next day.
4. Thaw one vial of ES cells in 37°C water bath, wash with ES medium, and transfer onto the feeder plate.
5. Change medium every 24 h until the cells are confluent (*see* **Note 3**).
6. Trypsinize confluent cells with 1 mL trypsin/ethylenediaminetetraacetic acid for 5 min and stop trypsin action by adding 5 mL ES medium.
7. Disperse cells to single-cell suspension by pipetting 10–15 times and transfer cells into a 15-mL tube.
8. Spin cells at 1000*g* for 5 min followed by resuspension in 10 mL PF medium.
9. Place cells on ice for 10 min to allow feeder cells to settle.

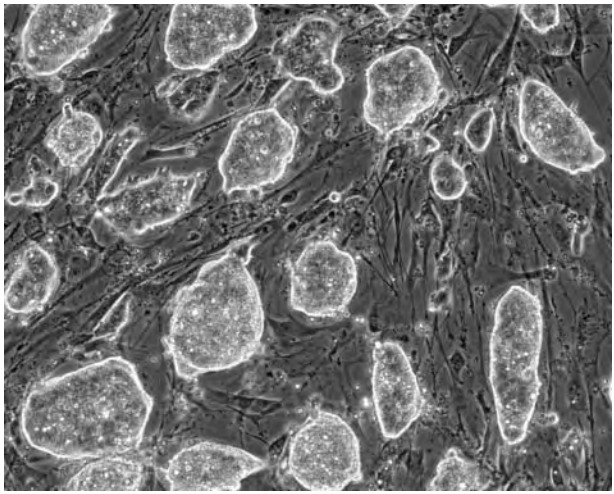

Fig. 1. Embryonic stem cells after 48 h of culture. Individual colonies can be seen growing over a monolayer of feeder cells. Each colony contains about 400–500 cells and is recognizable by distinct shiny edges. (×100 magnification.)

10. Collect top 8 mL cell suspension followed by another round of 10 min sedimentation and collection of top 6 mL to remove feeder cells completely.
11. Spin and wash cells at least twice in PF medium followed by resuspension in 5 mL fresh medium; check cell concentration by hemocytometer.

3.2. Electroporation of DNA Into ES Cells

ES cells are prepared to contain selectable marker gene such as neo or EGFP (*see* **Note 4**).

1. Prepare ES cells as described in **Subheading 3.1.**
2. Transfer 1–2 × 10^6 cells into a 15-mL tube and spin at 1000*g* in a microfuge centrifuge.
3. Wash pellet in 1X PBS once and resuspend cells into 700 µL PBS.
4. Dissolve 35 µg EGFP DNA in 100 µL 1X PBS.
5. Mix DNA with ES cells followed by transfer of all the contents to an electroporation vial.
6. Keep cells on ice for 10 min followed by electroporation using Bio-Rad Pulsar II at 250 V and 500 µF.
7. Allow cells to stand at room temperature for 5 min and transfer equal amounts onto four 100-mm plates with feeder cells.
8. Add 12 mL ES medium and allow cells to settle overnight.
9. Change medium the next day.
10. After 48 h, add fresh ES medium with 150–250 µg/mL G418.
11. After 8–10 d, individual colonies of ES cells can be seen spread throughout the plate.
12. Scrape colonies individually and transfer to 96-well plate with 50 µL trypsin.
13. After 4 min, add 100 µL ES medium and transfer contents into a 24-well feeder-coated plate and ES medium.
14. Collect approx 50–100 colonies and culture until the cells are confluent. Normally, it takes about 3–4 d.

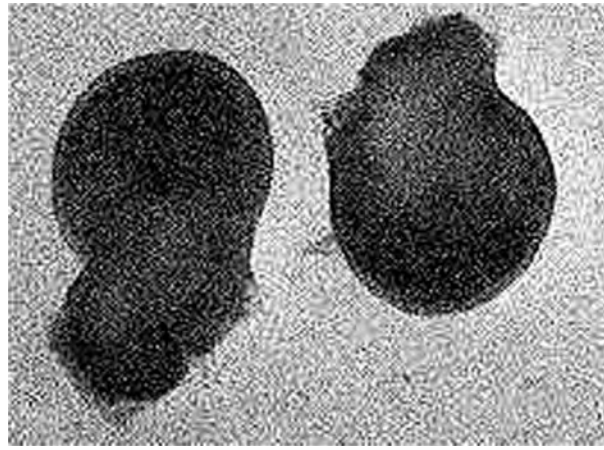

Fig. 2. Limb buds of E11.5 embryos. The limb buds appear as podlike structures. Limb buds from several embryos are pooled for the preparation of limb bud progenitor cells.

15. At confluency, trypsinize the cells and freeze half the cells in 10% dimethyl sulfoxide in ES medium and use the other half for DNA analysis.
16. Check for the presence of neo gene by PCR or by EGFP fluorescence using an ultraviolet microscope. The integrity of the DNA cells may be confirmed by Southern blot analysis.

3.3. Preparation of Limb Bud Progenitor Cells

The progenitor cells are obtained from limb buds of E11.5 embryos. Adult 5- to 6-wk-old females are mated with normal 10- to 12-wk-old stud males (*see* **Note 5**).

1. Set up two or three breeding pairs (*see* **Note 5**).
2. Verify the presence of vaginal plugs the following morning. The day of plug is E0.5.
3. Sacrifice pregnant females at 11 d by CO_2 asphyxiation. The embryos will be approx at E11–E11.5.
4. Collect uterine horns from the females and place in a 10-mm Petri dish with 1X PBS.
5. Cut the uterine wall and dispense embryos in a fresh Petri dish with PBS.
6. Wash embryos extensively to remove blood using a fresh Petri dish each time.
7. Excise limb buds from each embryo (**Fig. 2**).
8. Pooled limb buds from 8–10 embryos are trypsinized in 0.25% trypsin-ethylenediaminetetraacetic acid for 5–6 min followed by triturition in 10 mL PF medium to prepare a single-cell suspension.
9. Wash cells twice and resuspend in 10 mL PF medium.
10. Allow cells to stand for 3–5 min for tissue pieces to settle.
11. Transfer top 8 mL into a fresh tube and pellet cells by centrifugation.
12. Resuspend pellet in 5 mL PF medium and check cell concentration by hemocytometer.

3.4. Co-Culture of ES Cells and Progenitor Cells

To induce differentiation, ES cells are co-cultured with 25% limb bud progenitor cells (LBPCs) in high-density ($10–20 \times 10^6$ cells/mL) micromass cultures ***(9)***.

1. Transfer 1.5×10^5 ES cells and 50,000 LBPC ES cells in 500 µL PF medium in an Eppendorf tube.

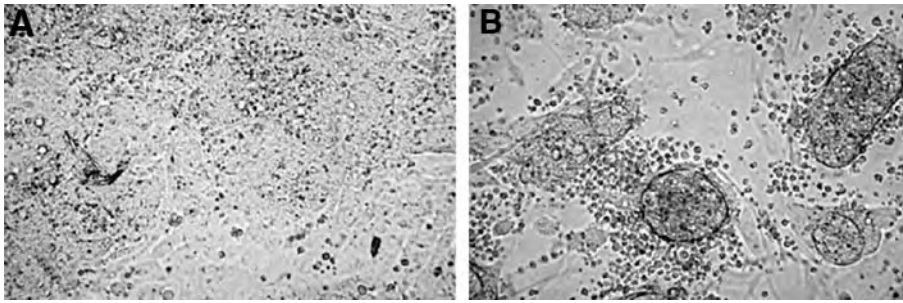

Fig. 3. Co-culture of embryonic stem (ES) cells with limb bud progenitor cells (LBPCs). ES cells were co-cultured with 25% LBPCs in high-density micromass culture (approx 5.0–10.0 × 10^6 cells/mL). The mixed cells form a uniform flat layer of cells after 24–48 h (**A**) as compared to pure ES cells, which form only multicellular colonies typical of undifferentiated cells (**B**). (×100 magnification.)

2. Mix cells on a lab rotator for 15 min (50–100 rpm) followed by resting the cells for 15 min in 37°C CO_2 incubator.
3. Repeat **step 2** twice and pellet cells in microfuge centrifuge at 1000*g*.
4. Resuspend cells in 20–25 µL PF medium and transfer total contents to four-well Nunc plate.
5. Parallel cultures of pure ES cells and LBPCs are processed separately as negative and positive controls, respectively.
6. After 2 h, cover the cells with approx 800 µL PF medium.
7. After overnight culture, ES cells form typical multicellular colonies (**Fig. 3A**), whereas co-cultured cells appear as a sheet of flat cells (**Fig. 3B**).
8. Change medium every other day.
9. After 4 d, treat the cells with 100 µg/mL G418 to select against LBPCs.
10. Differentiation of cells is monitored by the appearance of aggregates of cells (**Fig. 4A**, LBPCs; **Fig. 4B**, ES cells) (*see* **Note 6**).
11. The cells can be harvested at different time intervals for specific analyses.
12. For cell number, the aggregates and surrounding cells are scraped and counted with a hemocytometer after dissociation with trypsin and 2 mg/mL collagenase.

3.5. Alcian Blue Staining of Differentiated Cells

Terminally differentiated chondrocytes express cartilage-specific sulfated proteoglycans that stain positive with the alcian blue. Fully differentiated chondrocytes can be stained with alcian blue after 14 d.

1. Wash cells twice with 1X PBS.
2. Fix cells in 100% ethanol for 1 h.
3. Stain cells for 2–4 h with dye followed by washing twice with 100% ethanol.
4. Clarify cells with 80% glycerol solution (**Fig. 5**).

3.6. Reverse Transcriptase Polymerase Chain Reaction Analysis

G418-treated micromass cells are collected at different intervals by trypsinization, and total RNA is isolated. Reverse transcriptase (RT) PCR analysis is carried out using

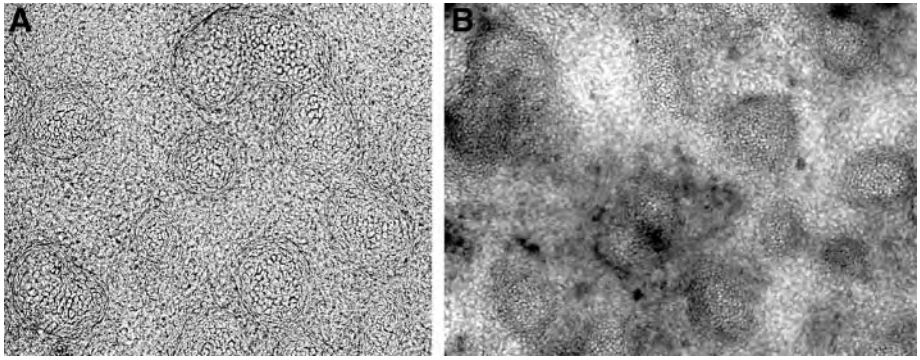

Fig. 4. Differentiation of embryonic stem (ES) cells into chondrocytes. High-density micromass cultures were established separately for (**A**) pure limb bud mesenchymal cells and (**B**) the mixed limb bud progenitor cells (LBPCs) and ES cells. Pure LBPCs form aggregates of condensed cells within 3–4 d. These aggregates develop into nodules of differentiated cells after 10–12 d of culture (**A**). Each nodule is usually surrounded by a unicellular layer of perichondrial cells. ES cells were co-cultured with LBPCs. After 4 d, the cells were treated with G418. The cells form aggregates after 7–8 d, approx 3–4 d later than that seen in pure LBPC cultures. These aggregates develop into nodules of chondrocytes after 14–15 d (**B**). Unlike pure LBPCs, the nodules in mixed cultures are surrounded by a small number of indistinct cells (dark-looking cells). These cells represent ES cells that failed to differentiate into chondrocytes. Cultures of pure ES cells did not form nodules (not shown). (×100 magnification.)

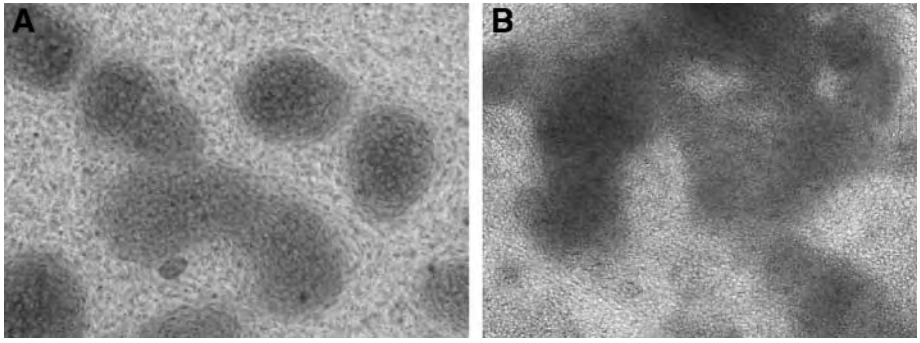

Fig. 5. Alcian blue staining of differentiated cells. Embryonic stem (ES) cells were co-cultured with 25% limb bud progenitor cells (LBPCs) followed by G418 treatment after 4 d of culture. After 15 d (i.e., about 19 d of culture), the cells were stained with alcian blue. In general, the nodules formed by differentiated ES cells exhibit diffused boundaries as compared to normal LBPCs. **(B)** Densely packed and overlapping nodules formed by the differentiated cells that stained positive with the dye. Staining of pure LBPC cultures shows individual nodules stained with the dye **(A)**. No alcian blue staining was observed in parallel culture of pure ES cells (not shown). (×100 magnification.)

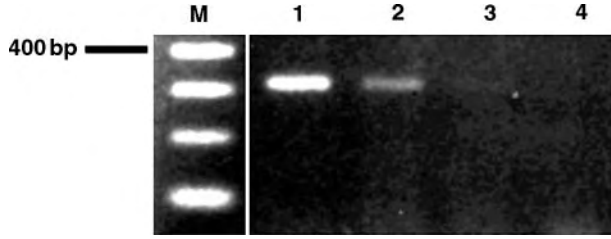

Fig. 6. Expression transcription factor Oct4. Undifferentiated embryonic stem (ES) cells express transcription factor Oct4, which disappears as the cells differentiate. Reverse transcriptase polymerase chain reaction analysis showed that undifferentiated ES cells express transcription factor Oct4 (**lane 1**). After 7 d, the expression of Oct4 decreased dramatically (**lane 2**) and disappeared completely in differentiated cells at d 19 (**lane 3**). Day 1 LBPCs did not show expression of Oct4 (**lane 4**).

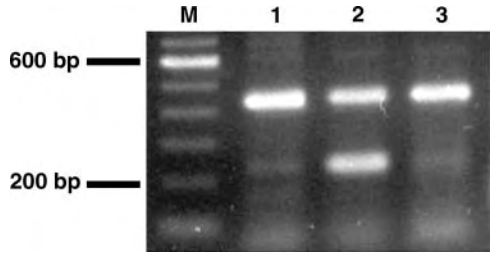

Fig. 7. Expression of chondrocyte-specific genes in differentiated cells. Embryonic stem (ES) cells were co-cultured with 25% limb bud progenitor cells (LBPCs) followed by G418 treatment after 4 d. The cells were harvested at d 7, and total RNA was isolated. **Lanes 1–3** show amplification of reverse-transcribed RNA for collagen type II. **Lane 1**, RNA from pure LBPCs at d 1; **lane 2**, RNA from LBPCs at d 7; **lane 3**, RNA from co-cultured and G418-treated cells at d 7. As expected, pure LBPCs on day of isolation showed the amplification of only type IIA-specific 489-bp fragment (**lane 1**), whereas the same cells at d 7 amplified DNA fragments for type IIA (489-bp) and type IIB (285-bp) transcripts, indicating the differentiation into chondrocytes (**lane 2**). The co-cultured cells, on the other hand, amplified only the 489-bp fragment specific for type IIA transcript (**lane 3**), similar to that in d-1 prechondrogenic cells. The ES cells did not show amplification of collagen type II at any stage (not shown).

specific primers for specific marker genes, for example, Oct4 for undifferentiated cells (**Fig. 6**) and collagen type II primers for differentiated cells (**Fig. 7**).

Collagen type II is the most abundant protein in the extracellular matrix of cartilage. It is expressed in two forms because of alternate splicing of exon 2 of the mRNA ***(10)***. The prechondrogenic mesenchymal cells express exclusively the unspliced form (i.e., type IIA transcript), whereas mature chondrocytes contain type IIB transcript in which exon 2 is spliced out. Co-cultured ES cells and LBPCs are treated with 100 µg/mL G418 at d 4 to eliminate limb bud-derived cells. After 3 d of treatment (i.e., after 7 d of culture) (*see* **Note 7**), total RNA is tested by RT-PCR analysis using primers specific for type IIA (489 bp) and type IIB (285 bp) transcripts (**Fig. 7**).

Primers for hypoxanthine phosphoribosyltransferase are used as a control for quantity of RNA. The PCR conditions are 60°C for 30 s; 72°C for 90 s; and 94°C for 30 s for 35–40 cycles. The amplified fragments are separated on 2% agarose gels.

3.7. Differential Gene Expression Analysis

For analyses of differentially expressed genes during cell fate determination, GFP-positive ES cells are mixed with normal LBPCs. After thorough mixing, the cells are plated as a micromass culture. After different intervals of time, the cells are trypsinized and separated by a fluorescent-activated cell sorter. The induced ES cells can be used directly for RNA isolation.

1. Mix 1.5×10^6 GFP-positive ES cells with 500,000 LBPCs (25%) from normal embryos.
2. After thorough mixing (*see* **Subheading 3.3.**, **steps 1–4**), resuspend cells in 100 µL PF medium.
3. Transfer 25 µL cell suspension to each well in a four-well plate.
4. Change medium every other day.
5. Harvest cells at specific intervals, such as 1, 2, 3, and 4 d, by trypsinization.
6. Separate induced ES cells in fluorescent-activated cell sorter (*see* **Note 6**).
7. The induced ES cells can be used directly for RNA isolation for differential gene expression analysis by microarray analysis.

4. Notes

1. Feeder cells are fibroblasts that are mitotically inactivated before preparing plates. The cells are treated with 10 µg/mL mitomycin C for 2 h. The cells are washed extensively in PF culture medium and are used directly to prepare feeder plates. Alternatively, the cells are irradiated at 4000–6000 rad, aliquoted as 1×10^6 cells, and stored in liquid nitrogen.
2. Several different ES cell lines are available from different laboratories, including commercial sources. R1 cells were obtained from Dr. A. Nagy. The lines have been established from the ICM of blastocysts. ES cells can be maintained indefinitely under specialized culture conditions, such as growth over feeder cells and supplementing medium with LIF. In general, ES cells require about 2000 IU LIF/mL. The feeder cells provide about 1000 IU; the rest is provided by supplementation.
3. For ES cell culture, the medium must be changed every day, and the cells must be split every 48–72 h; otherwise, the cells start to differentiate. The cells should be trypsinized before the colonies become too large. After trypsinization, the feeder cells can be separated from ES cells by letting the suspension stand. Feeder cells settle faster because they are heavier than ES cells, leaving pure ES cells on the top. The cells at the bottom also contain some ES cells. These cells can be saved for future use.
4. ES cells are electroporated with pEGFPN1 plasmid (Clonetech Inc.), which contains neo and EGFP gene. The cells are selected with 150–250 µg/mL G418, and the colonies with high fluorescence activity are collected and expanded individually.
5. To obtain E11.5 embryos, 5- to 6-wk-old normal females are mated with normal stud males. Usually, two or three females are mated with each male late in the evening. The pregnant females are checked by the presence of cream-colored vaginal plugs formed because of the hardening of secretions in the semen. The day of mating is considered E0.5.
6. The pure mesenchymal cells from limb buds form aggregates or nodules of differentiating cells after 3–4 d of culture. On the other hand, condensations in induced ES cells appear

after about 7 d, which suggests that the first 3–4 d are required for induction of ES cells into chondrogenic lineage.

7. The expression of the 489-bp band is seen after about 7 d in the induced cells, suggesting that the first 3–4 d are sufficient for the induction into chondrocytes by the LBPCs, indicating a stage equivalent to prechondrogenic cells.

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13

Derivation and Characterization of Chondrocytes From Embryonic Stem Cells In Vitro

Jan Kramer, Gunnar Hargus, and Jürgen Rohwedel

Summary

The model system of embryonic stem (ES) cell differentiation in vitro via cellular aggregates (embryoid bodies, EBs) can be used to analyze cell differentiation from a pluripotent stem cell via progenitor cells up to terminally differentiated cell types. ES cells are known to be pluripotent; they have the capacity to differentiate into any cell lineage of the three germ layers. Using various ES cell lines, we characterized chondrogenic and osteogenic differentiation in EBs by histochemical staining, immunostaining, mRNA-*in situ* hybridization, and reverse transcriptase polymerase chain reaction analysis. Here, we describe in detail our established protocols to analyze chondrogenic differentiation of ES cells. We summarize different ways to modulate ES cell-derived chondrogenic differentiation.

Key Words: Chondrogenic differentiation; embryonic stem cells; osteogenic differentiation.

1. Introduction

Embryonic stem (ES) cells of the mouse are generated from the inner cell mass of the blastocyst *(1,2)*. Because of their origin, ES cells are known to be pluripotent. In vitro, ES cells cultivated as cellular aggregates (embryoid bodies, EBs), spontaneously differentiate into various cell types of all three germ layers *(3)*. Our previous results demonstrated that this pluripotency also includes the ability of ES cells to differentiate into chondrocytes and osteoblasts *(4,5)*. ES cell-derived chondrogenic differentiation in vitro closely recapitulates cellular differentiation processes in vivo. In general, in vitro differentiation of mouse ES cells has been used as a model system to analyze cell differentiation from a pluripotent stem cell via progenitor cells up to terminally differentiated and specialized cell types (for reviews, *see* **refs.** *6* and *7*).

1.1. Differentiation of ES Cells Into the Chondrogenic and Osteogenic Lineage In Vitro

Using the ES cell line D3, we analyzed chondrogenic and osteogenic differentiation by histochemical staining, immunostaining, mRNA-*in situ* hybridization, and reverse

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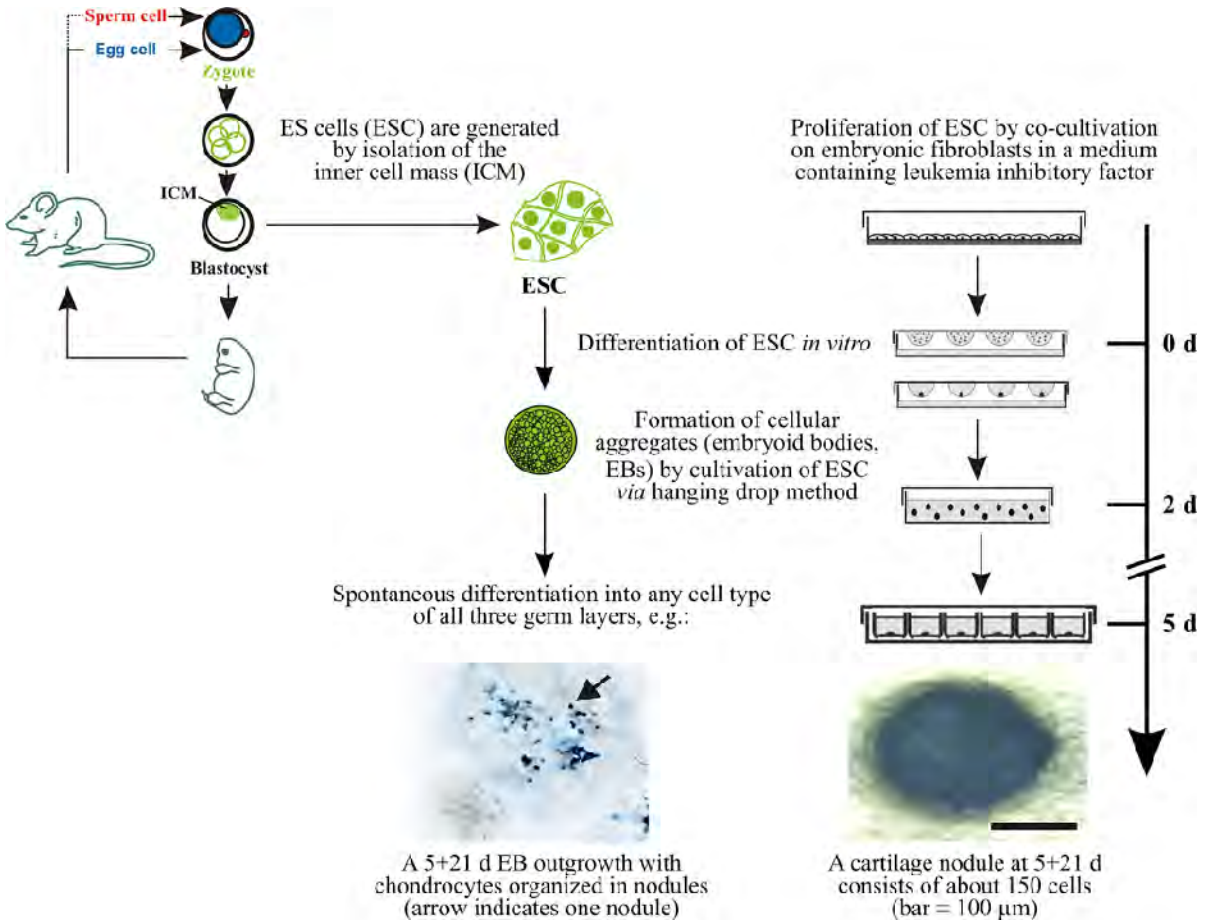
Sperm cell
Egg cell
Zygote
ICM
Blastocyst
ES cells (ESC) are generated by isolation of the inner cell mass (ICM)
ESC
Proliferation of ESC by co-cultivation on embryonic fibroblasts in a medium containing leukemia inhibitory factor
Differentiation of ESC *in vitro*
0 d
2 d
5 d
Formation of cellular aggregates (embryoid bodies, EBs) by cultivation of ESC *via* hanging drop method
Spontaneous differentiation into any cell type of all three germ layers, e.g.:
A 5+21 d EB outgrowth with chondrocytes organized in nodules (arrow indicates one nodule)
A cartilage nodule at 5+21 d consists of about 150 cells (bar = 100 μm)

Fig. 1.

transcriptase polymerase chain reaction (RT-PCR) techniques *(4)*. ES cells were differentiated by cultivation via EBs generated by hanging drops (**Fig. 1**). The major steps are: (1) generation of EBs by hanging drop cultivation of ES cells for 2 d (0–2 d); (2) cultivation of the EBs in suspension for 3 d (2–5 d); and (3) plating of the EBs at the fifth day of differentiation (5 d) followed by further cultivation up to 40 d (5 + 40 d).

We found that chondrocytes were organized in nodular structures that developed from mesenchymal condensations. Deeply alcian blue-stained areas were detected containing collagenous matrix in EB outgrowths (**Fig. 1**). This was confirmed by immunostaining for collagen II and X. We called these delimited areas with a distinct condensed group of cells *nodules*. Semiquantitative RT-PCR analysis demonstrated that genes encoding transcription factors involved in mesenchymal differentiation, such as Pax-1, scleraxis, and Sox-9, as well as extracellular matrix proteins of cartilage tissue such as aggrecan and collagen II, were expressed during ES cell differentiation *(4)*. ES cell-derived chondrogenic cells progressively developed into hypertrophic and calcifying cells *(5)*.

As indicated by loss of Alcian blue staining, the composition of the extracellular matrix in the nodules changed during differentiation. This was confirmed by immunostaining. At late differentiation stages, the cells localized in nodules expressed genes such as collagen X, osteopontin, bone sialoprotein, osteocalcin, and *cbfa*-1, characteristic for hypertrophic and calcifying chondrocytes. Finally, at a terminal differentiation stage the nodules stained positive for alkaline phosphatase (AP). The osteogenic cells appeared either by transdifferentiation from hypertrophic chondrocytes organized in nodules or directly from osteoblast precursor cells. This direct differentiation of ES cells into osteoblastic and osteogenic cells, bypassing the chondrocytic stage, was indicated by a population of osteogenic cells differentiating as single-cell clusters.

1.2. Modulation of ES Cell-Derived Chondrogenic Differentiation

In contrast to other mesodermal cell types, such as cardiac muscle cells, the amount of cartilage nodules per EB was relatively low in the ES cell line D3 *(8)*. The number of chondrogenic nodules spontaneously appearing in the EB outgrowths was influenced by different parameters. The amount of ES cells used for EB formation was important for the development of cartilage nodules. Among the EBs prepared from 200, 500, and 800 cells, the highest number of chondrogenic nodules was detected in outgrowths of EBs derived from 800 cells.

Remarkable differences regarding the efficiency of chondrogenic differentiation were also observed among different ES cell lines *(9)*. For example, in EB outgrowths derived from ES cell line BLC6 the number of chondrogenic nodules was approx fivefold higher compared to those derived from ES cell line D3. Other parameters influencing the differentiation potential were the basic cultivation medium, the batch of serum, the time of EB cultivation in suspension, and the day of EB plating. For example, more

Fig. 1. Origin, cultivation, and differentiation of embryonic stem (ES) cells. ES cells are cultivated as permanent cell lines of pluripotent cells isolated from the inner cell mass of blastocysts. For differentiation, cell aggregates are prepared using the hanging drop method. Cells of all three germ layers can be detected in embryoid body (EB) outgrowths. Cartilage nodules appear approx 14 d after EB plating and can be stained with Alcian blue. (Please *see* the companion CD for the color versions for this figure.)

nodules were detected in outgrowths of EBs cultivated in Dulbecco's modified Eagle's medium (DMEM) compared to EBs cultivated in Iscove's modified Dulbecco's medium *(8)*. Previous studies showed that optimal differentiation into the mesodermal direction could be obtained by cultivation in suspension for 3 d and plating at the fifth day of differentiation *(10,11)*.

Growth factors and signaling molecules might be useful to influence the efficiency of differentiation. We found that ES cell-derived chondrogenesis is modulated by growth factors of the transforming growth factor (TGF)-β family *(4)*. Treatment of EBs with 2 ng/mL TGF-β_1 for the entire differentiation period (from 1 d up to 5 + 35 d) resulted in unaltered or slightly reduced ES cell-derived chondrogenic differentiation. However, if bone morphogenetic protein (BMP)-2 (at a concentration of 2 ng/mL) or BMP-4 (at a concentration of 10 ng/mL) was applied to the EBs during the entire cultivation time (from 1 d up to 5 + 35 d), then the development of chondrogenic cells organized in nodules increased approx 2.5-fold. No obvious differences in the size or growth rate of EBs cultivated in the presence or absence of growth factors were observed. Furthermore, BMP-2-enhanced stage dependently the chondrogenic differentiation of ES cells. The effect of BMP-2 to increase chondrogenic differentiation was limited to a time window corresponding to the cultivation step of the EBs in suspension (2–5 d).

These results are in line with studies demonstrating the sensitivity of this period for the influence of differentiation factors (e.g., retinoic acid) on other mesodermal cell types (e.g., cardiogenic and skeletal muscle and adipogenic cells) *(12,13)*; for reviews, *see* **refs.** *13* and *14*. In addition, it has been shown previously that this early stage of ES cell differentiation is a decisive period of early mesodermal development *(15,16)*. It has also been shown that differentiation of murine ES cells toward the osteoblast lineage can be enhanced by supplementing media with ascorbic acid, β-glycerophosphate, or dexamethasone/retinoic acid or by co-culture with fetal murine osteoblasts *(17)*.

1.3. Isolation and Characterization of Chondrocytes From EBs

To analyze their differentiation behavior in culture, chondrocytes were isolated from nodules by microdissection and collagenase treatment *(5)* (**Fig. 2**). Initially, these isolated cells showed dedifferentiation in culture but later redifferentiated into mature chondrocytes as demonstrated by RT-PCR and immunostaining for collagen markers (**Fig. 3**) as described here. However, we observed additional cell types, such as adipogenic, epithelial, and skeletal muscle cells in the cultures *(5)*. This was demonstrated by Sudan III staining or by immunostaining for cytokeratin and sarcomeric α-actinin, respectively. Obviously, the isolated chondrogenic cells showed a distinct differentiation plasticity *(5)*, and it became evident that transdifferentiation of dedifferentiated chondrocytes at least into adipogenic cells occurred.

This differentiation plasticity may be a problem for future therapeutic application of ES cell-derived isolated chondrocytes in cell replacement approaches. We still consider ES cells a helpful tool for tissue engineering because of some unique features of these cells. First, it is possible to generate a nearly unlimited number of cells for transplantation in vitro because ES cells can almost indefinitely divide and proliferate in culture under specific conditions *(18)*. Second, ES cells exhibit a well-characterized capacity of differentiation into cell types of all three germ layers in contrast to adult stem cells.

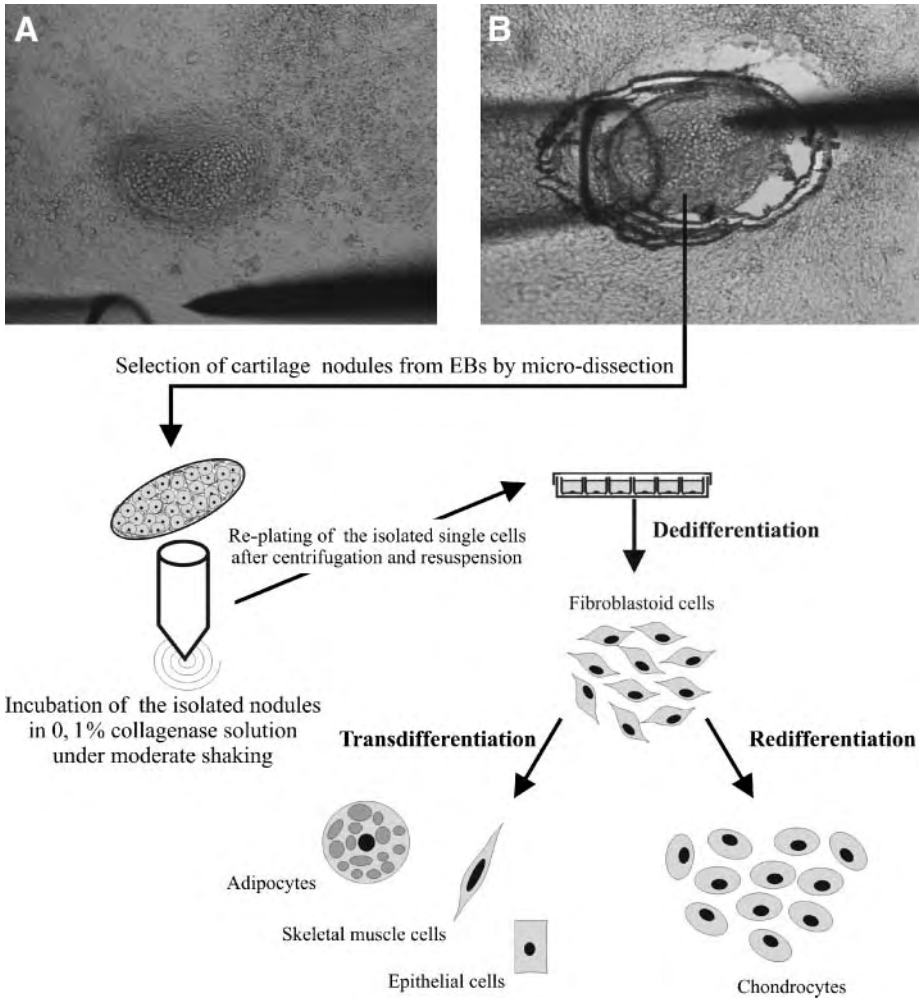

Fig. 2. To analyze their behavior in culture, chondrogenic cells can be isolated from embryoid bodies (EBs). Cartilage nodules can be detected in EBs of different cultivation stages by light microscopy because of their characteristic structure of dense, round cells surrounded by a fibrillar matrix (**A**). Our studies demonstrated previously that this matrix contains proteoglycans, collagen II, and other cartilage marker molecules. The nodules were cut from EB outgrowth using a microdissector (**B**). To obtain a single-cell suspension the selected nodules were treated with collagenase. The cells were replated at high density and analyzed for their differentiation behavior by histochemical staining, immunohistochemistry techniques, and reverse transcriptase polymerase chain reaction. At first, the isolated single cells dedifferentiated, but later they redifferentiated into mature chondrocytes. Again, cartilage nodule formation could be detected in the culture of the isolated cells. In addition, a few other cell types (e.g., adipogenic, epithelial, and skeletal muscle cells) can be observed in the cultures, suggesting a differentiation plasticity of the dedifferentiated embryonic stem cell-derived chondrogenic cell types.

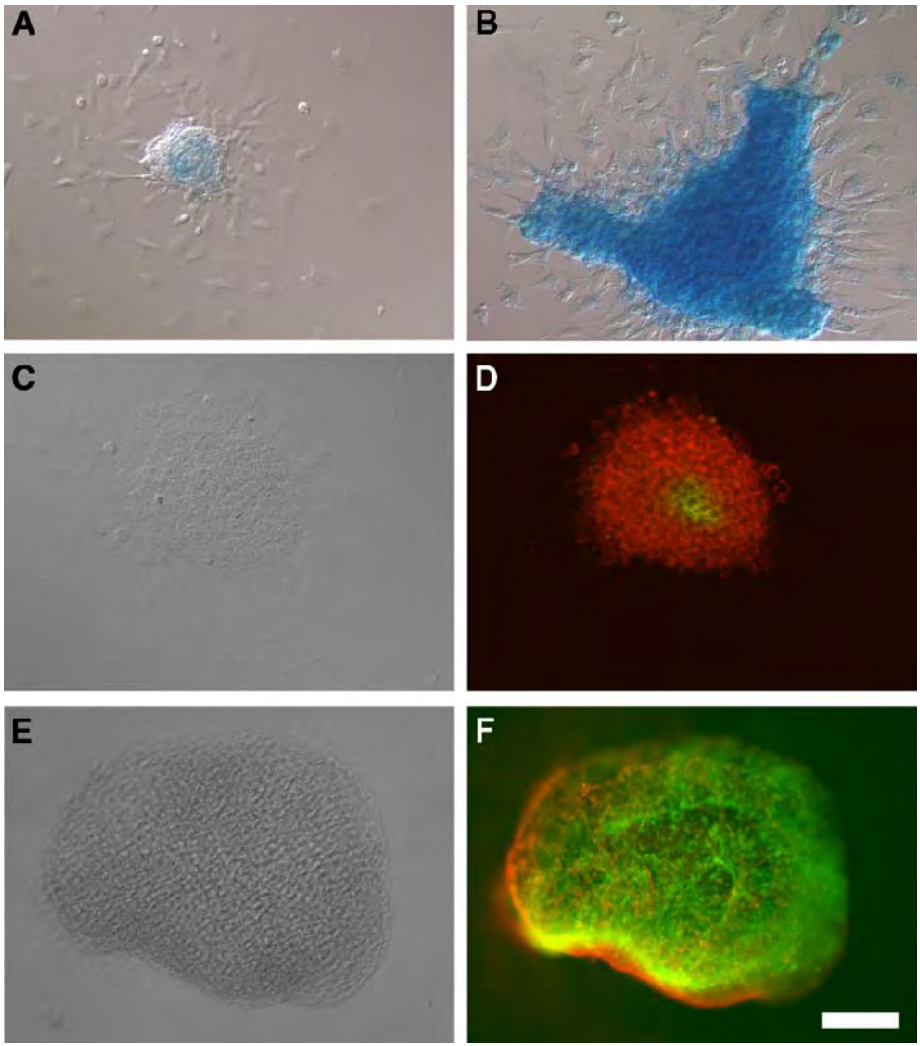

Fig. 3. Chondrogenic cells isolated from cartilage nodules in embryoid body outgrowths initially dedifferentiate but redifferentiate in culture. Chondrocytes can be isolated from embryonic stem cell-derived cartilage nodules and analyzed for their differentiation behavior. Initially, the single cells dedifferentiate, characterized by fibroblastoid morphology and a loss of collagen II expression, replaced by an upregulated expression of collagen I. Redifferentiation of the cells into chondrocytes is indicated by the formation of nodular structures. These nodules stain positive with alcian blue (**A**) and grow during further cultivation (**B**). During early nodule formation, the cells change their morphology from fibroblastoid to round (**C**), still expressing predominantly collagen I but only small amounts of collagen II (**D**). Later, the cells show the typical round morphology of mature chondrocytes (**E**), which express large amounts of collagen II (**F**). Bar = 100 µm. (Please *see* the companion CD for the color version for this figure.)

However, selection of specific cell types from EBs is still an absolutely necessary requirement before ES cells can be discussed for replacement therapies. For example, ES cells formed teratomas and destroyed the ambient tissue if they were injected into murine knee joints in an undifferentiated stage *(19)*. We suggest a selection method of ES cell-derived chondrocytes based on genetic manipulation as a suitable tool to isolate a distinct cell type from EBs. Therefore, we established a selection construct carrying the green fluorescent protein as a reporter expressed under the control of the cartilage-specific collagen II promoter. After transfection of ES cells with this construct and enhanced differentiation into chondrocytes, the chondrocytes express the reporter gene and can be isolated specifically from EBs by fluorescent-activated cell sorting. This selection strategy may be suitable to overcome the problem of differentiation plasticity of the isolated chondrocytes.

1.4. Deciphering Molecular Mechanisms of Chondrogenesis by Using Genetically Modified ES Cells

ES cells are used in vivo to study the phenotypic consequences of a gene mutation via gene targeting in mice because, after injection of genetically modified ES cells into blastocysts, these cells take part in the development of all somatic tissues, most importantly into germ cells *(20)*. However, it is possible that embryos carrying a homozygous knockout mutation die shortly after implantation. In this case, in vitro differentiation of homozygous knockout ES cells is an excellent alternative approach to analyze the consequences of a loss-of-function mutation at the cellular level.

We used the model system of ES cell-derived chondrogenic differentiation in vitro to characterize the function of the transcription factor Sox9 on chondrogenic differentiation *(21)*. Animals that carry a heterozygous gene knockout of Sox9 ($Sox9^{+/-}$) die soon after birth. Therefore, homozygous knockout mice have not yet been established. However, studies with heterozygous mice *(22)* and chimeras *(23)* showed that Sox9 plays an important role during chondrogenesis. In teratomas derived from $Sox9^{-/-}$ ES cells, no cartilage developed *(23)*. Furthermore, cartilage and bone was completely absent in the limbs of mice after conditional inactivation of the Sox9 gene in early mesenchymal limb bud cells, and severe chondrodysplasia was described when Sox9 was conditionally inactivated after mesenchymal condensations had formed *(24)*. We tested the homozygous Sox9-deficient ES cells for their cartilage differentiation and found that $Sox9^{-/-}$ cells formed mesenchymal condensations but failed to develop into cartilage nodules.

Altogether, the ES cell-derived system of chondrogenic differentiation is convenient and helpful to analyze cellular events of chondrogenesis in vitro. In this review, we summarize some basic techniques of ES cell cultivation and differentiation into the chondrogenic direction in vitro. We describe protocols to characterize chondrogenic differentiation of ES cells by histochemical staining, immunofluorescence methods, and RT-PCR techniques. Moreover, we summarize our experience regarding methods to modulate ES cell-derived chondrogenic differentiation. Finally, we suggest a route to isolate chondrocytes from EBs efficiently to make them a possible tool for in vivo transplantation settings.

2. Materials

2.1. Tissue Culture

1. Phosphate-buffered saline (PBS): 10 g/L NaCl, 0.25 g/L KCl, 1.44 g/L $Na_2HPO_4 \cdot H_2O$, 0.25 g/L KH_2PO_4 in distilled water (dH_2O). It is important to dissolve sodium and potassium phosphates before adding the other ingredients. Adjust to pH 7.2 and sterilize by filtration.
2. 0.2% trypsin (Biochrom, Berlin, Germany; cat. no. L-2133) in PBS (trypsin solution). 0.02% ethylenediaminetetraacetic acid (EDTA) in PBS (EDTA solution). A 1:1 mixture of trypsin and EDTA solutions is used to trypsinize cells.
3. For gelatin coating of tissue culture plastic, a 1% gelatin (Fluka, Taufkirchen, Germany; cat. no. 48720) solution in PBS is prepared as stock solution; autoclave. Tissue culture dishes, microwell plates, and chamber slides are treated with a 0.1% gelatin solution (stock solution diluted 1:10 with PBS) 1 d before cell or EB plating. Incubate on the plates overnight at 4°C and aspirate immediately before use.
4. 2 mg mitomycin C (MMC) (Serva, Heidelberg, Germany; cat. no. 29805) in 10 mL PBS (MMC stock solution) is sterilized by microfiltration. To obtain a MMC working solution, 300 µL MMC stock solution is diluted in 6 mL of medium 1.
5. 7 µL of mercaptoethanol β-(ME) (Serva; cat. no. MB148-25ML) in 10 mL PBS (β-ME stock solution) are sterilized by microfiltration.
6. 24-well microwell plates, two-chamber Lab Tek chamber slides (21.3 × 20 mm/well), and 60- and 100-mm tissue culture dishes (Nunc, Wiesbaden, Germany) are used after coating with gelatin.
7. 100-mm uncoated bacteriological Petri dishes (Greiner, Frickenhausen, Germany).
8. 15- and 50-mL centrifugation tubes and 2-mL cryoconservation vials (Nunc).
9. Sterile dissection instruments (scissors, forceps, and a sieve with a pore diameter of about 0.5 mm).
10. A sterile 100-mL Erlenmeyer flask with a stirring bar and approx 50 glass pearls with a diameter of about 4 mm.
11. Sterile 0.2-µm microfilters (Sartorius, Göttingen, Germany; cat. no. 16534).
12. 2-, 5-, 10-, and 20-mL pipets and a micropipetor for sterile 100-µL filter tips (Eppendorf, Cologne, Germany).
13. For our studies, we used ES cell lines BLC6 *(25)*, D3 *(26)*, E14 *(27)*, and R1 *(28)*.

2.1.1. Media

1. DMEM (Life Technologies, Eggenstein, Germany; cat. no. 41965062).
2. Fetal calf serum (FCS) (Sigma, Taufenkirchen, Germany) to supplement medium: to select an appropriate FCS batch, ES cells are differentiated via EBs (*see* **Subheading 3.1.5.**) using media supplemented with different FCS batches and analyzed for their chondrogenic differentiation efficiency by Alcian blue staining (*see* **Subheading 3.2.1.1.**).
3. 2 m*M* L-glutamine (Life Technologies, cat. no. 25030-081) (1 mL per 100 mL medium).
4. 5×10^{-5} *M* β-ME (Serva, cat. no. 28625.01) (1 mL β-ME stock solution per 100 mL medium).
5. Nonessential amino acids (Life Technologies, cat. no. 11140-35) (1 mL per 100 mL medium).
6. Penicillin plus streptomycin (Life Technologies, cat. no. 15140-122) (1 mL per 100 mL medium).
7. Medium 1: DMEM supplemented with 15% heat-inactivated FCS (*see* **Note 1**) and **items 3–6**.
8. Medium 2: DMEM supplemented with 15% heat-inactivated FCS, **items 3–6**, and leukemia inhibitory factor (LIF) (5 ng/mL; Chemicon, Temecula, CA; cat. no. LIF2005).

9. Medium 3: DMEM supplemented with 20% FCS and 8% dimethyl sulfoxide (Sigma, cat. no. C-6164).
10. Medium 4: DMEM supplemented with 20% FCS and **items 3–6**.

2.1.2. Growth Factors

For activation of chondrogenic differentiation, the following growth factors were tested, with the concentration used and the tested application periods given in parentheses:

1. Retinoic acid (10^{-8} *M*: 0–2 d, 2–5 d, 5 to 5 + 4 d; Sigma, cat. no. R2625).
2. TGF-β_1 (2 ng/mL: 1 to 5 + 30 d; Tebu, PeproTech, cat. no. 100-21R).
3. Fibroblast growth factor (FGF)-2 (2 ng/mL: 2–5 d; Biomol, Plymouth Meeting, PA; cat. no. 50361-2).
4. BMP-2 (2 ng/mL: 1 to 5 + 30 d; 10 ng/mL: 0–2 d, 2–5 d, 5 to 5 + 4 d; a gift from Genetics Institute, Cambridge, MA).
5. BMP-4 (10 ng/mL: 1 to 5 + 30 d; a gift from Genetics Institute).
6. TGF-β_3 (10 ng/mL: 0–2 d, 2–5 d, 5 to 5 + 4 d; Tebu, cat. no. 100-36).

2.2. Histochemical Staining

1. PBS (**Subheading 2.1., item 1**).
2. 3.7% formaldehyde solution.
3. AP staining kit (Sigma, cat. no. 86-R).
4. Citrate formaldehyde solution: 2.5 mL sodium nitrite solution, 6.5 mL acetone, and 0.8 mL 37% formaldehyde.
5. AP staining solution: 1 mL AP solution, 1 mL sodium nitrite solution, 45 mL dH_2O, and 1 mL naphthol solution.
6. Alcian blue working solution (0.05%; pH 1.5): dissolve 0.25 g alcian blue (Sigma, cat. no. A-3157), 4.5 g sodium chloride, and 6.4 g magnesium chloride in 500 mL 3% acetic acid. Stir the solution for 2–3 h and clear by filtration.
7. 5% acetic acid solution.
8. 100 m*M* Tris-hydroxymethyl-aminomethane (pH 9.0; Boehringer Mannheim, Mannheim, Germany; cat. no. 0604203).
9. Alizarin red staining solution 1: 5% alizarin red (Sigma, cat. no. A-5533); adjust to pH 9.0 with sodium hydroxide.
10. Alizarin red staining solution 2 (pH 7.0): 0.5% alizarin red solution in phosphate buffer.
11. Vectashield mounting medium (Vector, Burlingame, CA; cat. no. H-1000).
12. Nail polish.
13. Von Kossa silver stain: dissolve 500 mg silver nitrate (Fluka, cat. no. 31630) in 10 mL dH_2O.
14. Von Kossa fixation solution: 1 g sodium thiosulfate (Merck, cat. no. IC191447.5) in 20 mL dH_2O.
15. Sudan III staining solution: 0.2–0.3% Sudan III (Sigma, cat. no. S-4136) in 70% ethanol. Heat the solution for 15 min to 60°C and perform sterile filtration.

2.3. Immunostaining

1. PBS (**Subheading 2.1., item 1**).
2. Methanol acetone solution: 42 mL methanol and 18 mL acetone.
3. 10% goat serum (Dianova, Hamburg, Germany; cat. no. 005000001) in PBS.
4. Vectashield mounting medium (Vector, cat. no. H-1000).
5. Nail polish.

6. Primary antibodies: the following monoclonal antibodies (MAbs) can be obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA (designation of the MAb, the PBS dilution, and a reference are given):
   a. Collagen II: II-II6B3, 1:20 *(29)*.
   b. Osteopontin: MPIIIB10_1, 1:10 *(30)*.
   c. Collagen X: X-AC9, 1:20 *(31)*.
   d. Bone sialoprotein I and II: WVID1(9C5), 1:10 *(30)*.

   Immunostaining for cartilage oligomeric matrix protein (COMP; 1:20) *(32)* (a kind gift of M. Paulsson, Köln, Germany) and for collagen I (1:100; Chemicon, Temecula, CA; cat. no. AB765P) are performed using polyclonal antisera. In addition, the MAbs for cytokeratin (cytokeratin 1, 4, 5, 6, 8, 10, 13, 18, 19; 1:100; Sigma, cat. no. C2562) and sarcomeric actinin (EA-53; 1:200; Sigma, cat. no. A7811) are used to characterize transdifferentiation of isolated chondrocytes.
7. Secondary antibodies (dilution in PBS): immunoglobulin G labeled with fluorescein isothiocyanate (FITC; 1:200) or Cy3 (1:400) (Dianova, cat. no. 111-015-144 and 115-165-062, respectively).
8. Fluorescence microscope AXIOSKOP (Zeiss, Oberkochen, Germany).

2.4. *mRNA* In Situ *Hybridization*

1. PBS (**Subheading 2.1., item 1**).
2. 4% (w/v) paraformaldehyde, 4% (w/v) sucrose in PBS.
3. 2X SSC, 0.2X SSC, and 0.1X SSC (20X SSC stock solution: 175.3 g NaCl and 88.2 g sodium citrate in 1000 mL dH_2O).
4. 50, 70, 95, and 100% ethanol.
5. Prehybridization buffer containing 5X SSC, 5X Denhardt's solution (50X Denhardt's stock solution: 5 g Ficoll, 5 g polyvinylpyrrolidone, 5 g bovine serum albumin in 500 mL dH_2O), 50% formamide, 250 µg/mL yeast-t-RNA (Sigma, cat. no. R8759), 250 µg/mL denatured salmon sperm DNA (Life Technologies, cat. no. 15632-011) and 4 m*M* EDTA.
6. Digoxigenin-labeled sense and antisense probes against scleraxis (1 ng/µL) or collagen X (1 ng/µL).
7. FITC-conjugated sheep F(ab) fragments against digoxigenin (Roche, Mannheim, Germany; cat. no. 1207741).
8. Cy3-conjugated goat antimouse secondary antibodies (Dianova, cat. no. 115-165-062) for indirect detection of collagen II, both diluted 1:800 in PBS.
9. Vectashield mounting medium (Vector, cat. no. H-1000).
10. Nail polish.
11. DIG RNA labeling mix kit including polymerases SP6 and T7 (Boehringer Mannheim, cat. no. 1175025).
12. TOPO II cloning kit (Invitrogen, Groningen, NL; cat. no. K4600-01).

2.5. Reverse Transcription and Polymerase Chain Reaction

1. RNeasy minikit (Qiagen, Hilden, Germany; cat. no. 74104).
2. Oligo-dT primer (Life Technologies, cat. no. 18418-012).
3. SuperScript II reverse transcriptase (Life Technologies, cat. no. 18064-014).
4. *Taq* DNA polymerase (Roche, cat. no. 1647679).
5. Primer (*see* **Table 1**).
6. 0.1% agarose gel.

Table 1
Oligonucleotide Sequences of Primers Used to Study Chondrogenic and Osteogenic Gene Expression During ES Cell Differentiation In Vitro by RT-PCR

Gene	Antisense primer	Sense primer	Ann. temp.	Fragment length
Scleraxis	5′-GTGGACCCTCCTCCTTCTAATTCG-3′	5′-GACCGCACCAACAGCGTGAA-3′	63°C	375 bp
Pax-1	5′-TTCTCGGTGTTTGAAGGTCATTGCCG-3′	5′-GATGGAAGACTGGGCGGGTGTGAA-3′	60°C	318 bp
Sox-9	5′-TCTTTCTTGTGCTGCACGCGC-3′	5′-TGGCAGACCAGTACCCGCATCT-3′	57°C	135 bp
Cbfa-1	5′-ATCCATCCACTCCACCACGC-3′	5′-AAGGGTCCACTCTGGCTTTGG-3′	63°C	371 bp
Aggrecan	5′-TCCTCTCCGGTGGCAAAGAAGTTG-3′	5′-CCAAGTTCCAGGGTCACTGTTACCG-3′	60°C	270 bp
Collagen II	5′-AGGGGTACCAGGTTCTCCATC-3′	5′-CTGCTCATCGCCGCGGTCCTA-3′	60°C	432 bp (A) 225 bp (B)
Collagen X	5′-ATGCCTTGTTCTCCTCTTACTGGA-3′	5′-CTTTCTGCTGCTAATGTTCTTGACC-3′	61°C	164 bp
Osteocalcin	5′-ATGCTACTGGACGCTGGAGGGT-3′	5′-GCGGTCTTCAAGCCATACTGGTC-3′	64°C	330 bp
β-Tubulin	5′-GGAACATAGCCGTAAACTGC-3′	5′-TCACTGTGCCTGAACTTACC-3′	54°C	317 bp
HPRT	5′-GCCTGTATCCAACACTTCG-3′	5′-AGCGTCGTGATTAGCGATG-3′	63°C	507 bp

Sequence-specific antisense and sense primer were used at different annealing temperatures (Ann. Temp.). The lengths of the amplified fragments are given. Primer used to detect collagen II expression amplified two fragments representing a juvenile (A) and adult (B) splice variant. The housekeeping genes β-tubulin or HPRT were used as internal standards.

2.6. Isolation of Chondrogenic Cells

1. Microscalpel or microdissector (Eppendorf, Hamburg, Germany).
2. Tissue-Tek OCT (Sakura Finetechnical, Tokyo, Japan; cat. no. 4583).
3. Kryostat (Leica, Bensheim, Germany).
4. Acetone for fixation.
5. PBS (**Subheading 2.1., item 1**).
6. 2% collagenase solution.

3. Methods

3.1. Tissue Culture

All cultivation procedures have to be done in a CO_2 incubator at 37°C in a humidified 5% CO_2 atmosphere.

3.1.1. Preparation and Cultivation of Embryonic Fibroblasts

Embryonic fibroblasts used as feeder layer cells for ES cell cultivation are prepared and cultivated according to an established method *(33)*.

1. Remove embryos at d 14 pc (*see* **Note 2**) from the uteri of pregnant mice and rinse under sterile conditions in PBS. Cut off the placenta and fetal membranes and remove head and liver using sharp scissors.
2. Rinse the remaining carcass first in PBS and then in a 0.2% trypsin solution.
3. Mince the embryonic tissue in 5 mL fresh 0.2% trypsin solution. Transfer to a sterile Erlenmeyer flask with glass pearls and stir for 25–45 min.
4. Filter the resulting cell suspension through a sterile sieve (*see* **Note 3**) and add 10 mL medium 1.
5. Spin the cells down at 180*g*, resuspend the pellet in 3 mL medium 1, and plate onto gelatin-coated 100-mm tissue culture dishes filled with 10 mL medium 1 (approx 2×10^6 cells isolated from about two embryos are plated per dish).
6. Culture feeder layer cells for 1–2 d and either trypsinize and freeze in medium 3 (one dish per freezing vial) or split 1:3 onto gelatin-coated 100-mm tissue culture dishes and cultivate for 1 d more in medium 1 for further use.
7. Use MMC for growth inactivation of the subcultured or frozen feeder cells (*see* **Subheading 3.1.2.**). Frozen cells are thawed rapidly, plated onto two gelatin-coated 100-mm tissue culture dishes, and cultivated overnight.

3.1.2. Treatment of Feeder Layer With MMC Before Co-Cultivation With ES Cells

1. For inactivation, incubate the cells with MMC working solution for about 2.5 h at 37°C in a CO_2 incubator.
2. After aspirating the MMC working solution, wash the cells three times with PBS. Trypsinize the cells.
3. Plate the cells onto gelatin-coated 60-mm culture dishes with 4 mL medium 1. A confluent monolayer is obtained using about 1.5×10^5 cells per 60-mm culture dish (*see* **Note 4**).

3.1.3. Cultivation of ES Cells

ES cells are maintained in the undifferentiated state by cultivation on feeder layer cells and under the influence of LIF. It is critical to subculture the ES cells carefully for

maintaining the undifferentiated ES cells in the pluripotent state (*see* **Note 5**). Therefore, ES cells should be subcultivated every 24 h (48 h maximum).

1. Replace medium 1 on the feeder layer by medium 2 at 2 h before the ES cells are thawed.
2. Thaw the vial with the ES cells rapidly at 37°C.
3. Dilute the cold cell suspension 1:10 with medium 2 and spin down for 5 min at 180*g*.
4. Resuspend the cell pellet in medium 2 and transfer onto the prepared feeder layer.
5. Replace medium 1 on the feeder layer 1–2 h before subcultivation by medium 2.
6. For subcultivation, remove medium 2 from the ES cells and wash the cells once with PBS.
7. Add 2 mL trypsin/EDTA solution to the ES cells and incubate for 30–60 s at room temperature. Observe the detachment of the cells under the microscope.
8. Suspend the cells in trypsin/EDTA solution, transfer them to a centrifuge tube with 10 mL medium 2, and spin down for 5 min at 180*g*.
9. Resuspend the cell pellet thoroughly in medium 2 using a 2-mL glass pipet.
10. Transfer the single-cell suspension 1:3 onto 60-mm tissue culture dishes with MMC-inactivated feeder layer.
11. Observe by microscopy (*see* **Note 5**).

3.1.4. Freezing of ES Cells

1. Trypsinize the cells and resuspend the cells in 10 mL medium 2.
2. Centrifuge for 5 min at 180*g* and resuspend the cells in 1.5 mL medium 3.
3. Transfer the single-cell suspension to a freezing vial.
4. Freeze the cells slowly at –80°C overnight (*see* **Note 6**) and transfer to liquid nitrogen.

3.1.5. In Vitro Differentiation of ES Cells Via EBs

ES cells were differentiated in vitro by cultivation as EBs generated by hanging drops (*see* **Fig. 1**).

3.1.5.1. Hanging Drop Cultivation of ES Cells (0–2 d)

1. Trypsinize the undifferentiated ES cells cultivated on feeder layer.
2. Centrifuge for 5 min at 180*g* in medium 4.
3. Resuspend in 2 mL medium 4 and determine the number of cells.
4. We used 800 ES cells per EB for differentiation into chondrogenic and osteogenic cells. Cells are diluted in medium 4 to obtain a cell suspension with 4×10^4 cells/mL (*see* **Note 7**).
5. Place 20-µL aliquots of the cell suspension on the inside of a 100 mm-bacteriological Petri dish lid using a micropipetor with sterile 100-µL filter tips (*see* **Note 8**).
6. The dish is filled with 10 mL PBS to avoid evaporation of the medium.
7. The hanging drops are cultivated for 2 d at 37°C (0–2 d).

3.1.5.2. Cultivation of EBs in Suspension (2–5 d)

1. On the second day of hanging drop culture, drops with the EBs are collected with 2 mL medium 4 using a 5-mL glass pipet.
2. Transfer into 8 mL medium 4 in a 100-mm bacteriological Petri dish. The bacteriological dish is not coated with gelatin. Two lids with about 50 drops per lid are collected in one dish.
3. EBs are cultivated in suspension for 3 d (2–5 d; *see* **Note 9**).

3.1.5.3. Plating of EBs (5 d)

1. On the fifth day of differentiation (5 d) EBs are plated onto gelatin-coated 60-mm tissue culture dishes (for histochemical staining and RT-PCR), chamber slides (for immunostaining, *in situ* hybridization, and AP staining; *see* **Note 10**), and microwell plates (for morphological analysis). After adding medium 4 (4 mL per 60-mm dish, 1.5 mL per chamber, and 1 mL per well), EBs are transferred using a micropipetor with sterile 100-µL filter tips. Plate 10 EBs/60-mm dish, 5 EBs per chamber, and 1 EB per well (*see* **Note 11**).
2. Initially, the medium is changed after 4 and 8 d.
3. During further cultivation steps up to 40 d after plating (5 + 40 d), the medium is renewed every second day.
4. To study the differentiation process, samples are analyzed every second day.

3.1.6. Isolation of Chondrocytes From EBs

To study their behavior in culture, chondrogenic cells were isolated from EBs by microdissection. EBs were cultivated as described in **Subheading 3.1.5.**, and chondrogenic nodules were cut from the EB outgrowths using either a microscalpel or a microdissector and collected in PBS (**Fig. 2**). To characterize the differentiation stage of these cells, samples of these nodules were investigated for expression of collagen marker proteins by immunostaining of cryosections *(5)*.

3.1.6.1. Characterization of Isolated Nodules

1. Select nodules by microdissection and embed in Tissue-Tek OCT.
2. Freeze the samples at (20°C. Prepare cryosections (10 µm) using a cryostat and plate onto Vectabond (Vector)-coated slides.
3. Dry the sections in air and fix in acetone for 10 min at –20°C.
4. Wash in PBS and perform indirect immunostaining after fixation as described in **Subheading 3.2.2.**

3.1.6.2. Preparation of Chondrocytes From Selected Cartilage Nodules

1. Incubate the selected nodules in 0.1% collagenase solution for 50 min at 37°C. This has to be done under moderate shaking to obtain a single-cell suspension.
2. Centrifuge the cells for 5 min at 180*g*, resuspend in medium 4, and plate at high density onto gelatin- or collagen II- (Sigma) coated 60-mm tissue culture dishes (at a density of 1–2 $\times 10^5$ cells) or onto chamber slides (at a density of 2.1×10^4 cells/well) for total RNA isolation or immunostaining and histochemical staining, respectively.

3.2. Analysis of ES Cell-Derived Chondrogenic and Osteogenic Differentiation

3.2.1. Histochemical Staining of EBs

We performed histochemical staining of the EBs throughout cultivation from 5 + 2 d up to about 5 + 40 d at regular intervals of about 2 d. EBs must be fixed before staining (except for Sudan III and AP staining).

1. Remove media and wash with PBS.
2. Add 3.7% formaldehyde solution for 30 min to the probes.
3. Remove formaldehyde and wash three times in PBS and once in distilled water.
4. View by light microscopy.

3.2.1.1. Alcian Blue Staining

EBs can be stained with alcian blue as a fast screen for chondrogenic nodules. Alcian blue selectively stains mucosubstances, particularly cartilage-specific proteoglycans.

1. EBs are cultivated on 60-mm culture dishes and are fixed as described in **Subheading 3.2.1.**
2. Add 4 mL alcian blue solution to the specimens overnight.
3. Remove the staining solution and wash for 30 s once with PBS and once with 5% acetic acid.
4. Wash one more time with PBS and add approx 5 mL PBS to avoid evaporation of the specimens.
5. View by light microscopy.

3.2.1.2. Alizarin Red Staining

Alizarin red forms chelate conjugates with bivalent cations (mainly with calcium).

1. EBs are cultivated on chamber slides and are fixed as described in **Subheading 3.2.1.**
2. Transfer the slides into Tris-hydroxymethyl-aminomethane buffer for 30 s.
3. Incubate the samples in pH 9.0 staining solution for 1 h. Remove the staining solution by rinsing twice with PBS.
4. Incubate the samples in pH 7.0 staining solution for 5 min. Remove the staining solution by rinsing twice with PBS. Embed and cover the slides with slips.
5. View by light microscopy.

3.2.1.3. von Kossa Staining

The von Kossa staining is caused by ion exchange (calcium ions are replaced by silver), which depends on the influence of daylight.

1. EBs are cultivated on 60-mm culture dishes and are fixed as described in **Subheading 3.2.1.**
2. Treat the EBs for 10–20 min with 5% silver nitrate solution.
3. Wash carefully twice with distilled water for 15 s each.
4. Fix the resulting staining with 5% sodium thiosulfate solution for 2 min.
5. View by light microscopy.

3.2.1.4. Sudan III Staining

Adipogenic differentiation of ES cells can be demonstrated by staining with Sudan III. The process of staining can be tracked directly by light microscopy.

1. EBs are cultivated on chamber slides. Do not fix the specimens.
2. Wash the cells with PBS for 15 s.
3. Add the Sudan III staining solution for 3 min.
4. View by light microscopy.

3.2.1.5. AP Staining

We used AP staining to detect osteogenic cells during later differentiation stages of EBs. EBs were cultivated on chamber slides.

1. Wash the cells twice for 15 s with PBS.
2. Add citrate formaldehyde solution for 30 s to fix the EBs.

3. Rinse the samples with distilled water.
4. Incubate the samples in AP staining solution for 15 min in the dark at room temperature.
5. Wash in distilled water for 2 min before and after counterstaining with hematoxylin solution for 2 min.
6. Rinse slides thoroughly in tap water, air-dry, and analyze microscopically.

3.2.2. Indirect Immunostaining of EBs for Expression Analysis of Cartilage-Specific Proteins

EBs were analyzed for differentiated cells by immunostaining. EB cultures are set up and maintained on chamber slides. Incubation is performed in a humidified chamber (*see* **Note 12**).

1. Rinse specimens twice in PBS.
2. Fix the cells for 5 min with methanol:acetone (7:3) at –20°C. Rinse three times in PBS.
3. Incubate the cells for 15 min in 10% goat serum at room temperature.
4. Incubate the specimens with the specific primary antibodies for 1 h at 37°C in a humidified chamber. Rinse three times with PBS.
5. Incubate the slides for 45 min at 37°C with FITC (1:200)- or Cy3 (1:400)-labeled immunoglobulin G depending on the origin of the primary antibody.
6. Wash the slides three times in PBS and once in distilled water.
7. Embed the specimens in Vectashield mounting medium and cover the slides with slips.
8. Perform microscopic analysis using a fluorescence microscope.

3.2.3. Whole-Mount Fluorescence In Situ *Hybridization for Scleraxis- or Collagen X-mRNA Coupled With Immunostaining for Collagen II*

For fluorescence mRNA *in situ* hybridization, a modified whole-mount procedure for EBs ***(15)*** is used. Samples of five EBs cultivated on chamber slides are analyzed at different developmental stages. For *in situ* hybridization and for immunostaining, the same humidified chamber is used (*see* **Note 12**).

3.2.3.1. Generation of mRNA Hybridization Probes

1. Amplify a fragment of scleraxis- or collagen X-complementary DNA from RNA isolated from 16 d pc mouse limb buds following the RT-PCR protocol and using the primer listed in **Table 1**.
2. Blunt-end clone the fragments into the plasmid vector pCR-BluntII-TOPO using the TOPO II cloning kit according to the manufacturer's protocol and verify the sequences by sequencing.
3. Synthesize digoxigenin-labeled RNA probes of either sense or antisense orientation of scleraxis or collagen X from linearized plasmids of the cloned complementary DNA fragments by in vitro transcription using the T7- or SP6-RNA polymerase and the DIG RNA labeling mix following the protocol supplied by the manufacturer.

3.2.3.2. *In Situ* Hybridization and Immunostaining

1. Rinse the chamber slides twice with PBS.
2. Fix the EBs with 4% (w/v) paraformaldehyde and 4% (w/v) sucrose in PBS for 20 min at room temperature.
3. Wash the cells twice in PBS for 5 min.

4. Incubate the specimens at 70°C in 2X SSC for 15 min.
5. Wash with PBS and 2X SSC for 3 min.
6. Repeat **steps 2–5**.
7. Subsequently dehydrate the cells at room temperature for 2 min each in 50, 70, and 95% ethanol and twice in 100% ethanol.
8. Perform prehybridization in prehybridization buffer for 3 h in a humid chamber at 45°C.
9. Perform hybridization with 500 µL/well digoxigenin-labeled sense and antisense probes against scleraxis (1 ng/µL) or collagen X (1 ng/µL) in prehybridization buffer without salmon sperm DNA at 45°C in a humidified chamber overnight.
10. Wash the specimens after hybridization at the next day twice with 2X SSC for 15 min, once with 0.2X SSC for 15 min, and twice with 0.1X SSC for 15 min at 45°C. Rinse in PBS.
11. Apply the monoclonal antibody II-II6B3 against collagen II (diluted 1:20 in PBS) to the cells and incubate in a humidified chamber for 1 h at 37°C.
12. Wash the cells three times with PBS at room temperature.
13. Add FITC-conjugated sheep F(ab) fragments against digoxigenin and Cy3-conjugated goat antimouse secondary antibodies for indirect detection of collagen II, both diluted 1:800 in PBS. Incubate for 1 h at 37°C.
14. Wash the slides three times in PBS and once in distilled water.
15. Embed in Vectashield mounting medium and cover the slides with slips.
16. Perform analysis with a fluorescence microscope.

3.2.4. Detection of Cartilage-Specific Gene Expression in EBs by RT-PCR

To analyze the expression of tissue-specific genes during EB cultivation, samples of 10 EBs were plated per 60-mm tissue culture dish. Negative and positive controls were always included (*see* **Note 13)**.

1. Wash the cells twice in PBS.
2. Isolate total RNA using the RNeasy minikit according the manufacturer's recommendations.
3. Determine the RNA concentrations by measuring the absorbance at 260 nm.
4. Reverse transcribe samples of 500 ng RNA using Oligo-dT primer and SuperScript II RT following the manufacturer's recommendations.
5. Use 1-µL aliquots from RT reactions for amplification of transcripts using primers (*see* **Table 1**) specific for the analyzed genes and *Taq* DNA polymerase according to the manufacturer's instructions. RT reactions are denatured for 2 min at 95°C, followed by 34–45 cycles of 40 s denaturation at 95°C, 40 s annealing at the primer-specific temperature, and 50 s elongation at 72°C. For RT-PCR analysis of scleraxis, we use an established method (***34,35***), including the oligonucelotide primer for hypoxanthine phosphoribosyltransferase (HPRT) and scleraxis in the same reaction.
6. Perform electrophoretic separation of PCR products in 2% agarose gels.
7. Analyze the fragments by computer-assisted densitometry in relation to HPRT or β-tubulin gene expression.

4. Notes

1. For heat inactivation, FCS is thawed at room temperature and incubated at 54°C for 30 min.
2. Mouse embryos 14 d pc are optimal for isolation of embryonic fibroblasts used as a feeder layer. However, embryos up to 16 d pc can be used.
3. We use a metal tea filter, which can be autoclaved.
4. A 1-d-old feeder layer is optimal. However, the growth-inactivated feeder cells can be used up to 4 d after preparation.

5. For cultivation of ES cells in the pluripotent state, it is important that the cells grow in distinct colonies with a clear contour and limited size. Remark that a single-cell status after resuspension is essential for efficient cultivation.
6. A simple styropor box can be used to avoid rapid cell freezing. Alternatively, we use a freezing container (Cryo 1°C freezing container; Nalgene, Rochester, NY; cat. no. 5100-0001).
7. For cell dilution, it is helpful to use a small beaker because it is easy to handle the 20-µL drop samples with a micropipetor under sterile conditions.
8. If using gloves for cell culture handling, then electrostatic charge of the Petri dish lid can cause the drops to flow together. Therefore, avoid touching the lids on the outside top.
9. It is important to select a batch of bacteriological Petri dishes that enables suspension culture. In some batches, the EBs attach even if the dishes are not coated with gelatin. If this happens, then it is sometimes possible to resuspend the EBs by carefully rinsing the dishes with medium 4.
10. We use Falcon chamber slides (Becton-Dickinson, Heidelberg, FRG; cat. no. 354102) because the chambers can be removed relatively easily.
11. After plating, the EBs need some time to attach. Therefore, the dishes should be handled very carefully up to 2 d after plating.
12. We use a 15-cm glass dish with a wet piece of filter paper covered with a lid as a humidified chamber.
13. RT-PCR: RNA from limb buds or limbs of mouse embryos 10 and 16 d pc was always used as positive controls. Distilled water was included as a negative control.

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14

Generation and Characterization of Cardiomyocytes Under Serum-Free Conditions

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Summary

In vitro culturing of mammalian cells provides an elegant platform to study cell signaling, interactions, and metabolism as well as proliferation and differentiation processes. Often, these cells are cultured and maintained in sera obtained from animals such as horses, cows, and rabbits. The sera used for this purpose fluctuates in composition from individual animals and, hence, influences the cellular growth and differentiation at different magnitudes. This poses a need to use a substitute for sera in cell culture systems to overcome the observed variations. Here, we present and compare protocols for culturing of embryonic stem (ES) cells in serum-free conditions, derivation of germ layers, and cardiac differentiation of ES cells in both serum-free and serum-containing culture conditions. Differentiated embryoid bodies by serum-free protocols produce significantly increased frequencies of clusters of cardiac cells beating stronger than found in serum-containing media. Therefore, we conclude that the use of serum replacement media (SRM) in our experiments led to more specific differentiation but reduced proliferation because these SRMs contained reduced essential substances like growth factors and hormones. Unlike serum media, SRMs have a well-defined composition and are highly reproducible. Hence, SRM will be the ideal substitute for serum-containing media.

Key Words: CGR8 cells; cardiomyocytes; differentiation markers; embryoid bodies; embryonic stem cells; germ layers; serum replacement medium.

1. Introduction

Embryonic stem (ES) cells can be used for the generation of transgenic animals, as a source for tissue renewal, or as an in vitro model of embryonic development *(1–4)*. Murine ES cells are derived from the inner cell mass (ICM) of the preimplantation mouse embryo *(5)* and can be cultured in vitro while retaining the functional attributes of their pluripotency. The presence of the leukemia inhibitory factor (LIF) allows ES cells in vitro to continue proliferating indefinitely without differentiating as a stable cell

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line (**Fig. 1**) ***(6–9)***. In the absence of LIF, ES cells undergo differentiation and generate the three types of germ layers (endoderm, ectoderm, and mesoderm) in addition to the well-defined tissues derived from each of the three germ layers during their terminal differentiation, for example, cardiomyocytes (CMs) or nerve cells (for review, *see* **refs.** ***5*** and ***10–14***).

Like other mammalian cell lines, ES cells are cultured in a medium supplemented by serum (mostly fetal bovine serum [FBS] or fetal calf serum [FCS]), the composition of which is not well defined. The concentration of hormones and growth factors in serum varies significantly from batch to batch, depending on the status of the donor animal at the time of collection. Hence, composition of the sera used in the ES culture is not authentic and varies significantly in terms of the relative concentration of hormones and growth factors contained within. Consequently, the magnitude of the effects imparted on cellular growth and differentiation is entirely dictated by the serum used and therefore poses a difficulty in getting reproducible results. This necessitates an alternative substitute to replace serum from the ES culture medium to circumvent both significant and subtle variations encountered in the serum-containing medium.

We present here the following protocols for culturing ES cells, generation of embryoid bodies (EBs) ***(15)***, and their subsequent differentiation into beating CMs in serum-free conditions (**Fig. 2**): (1) differentiation of EBs into CMs in serum replacement medium 2 (SRM2); (2) serum-free culture of ES cells and subsequent generation of EBs and their differentiation into CMs in knockout serum replacement medium (k-o SRM)/knockout Dulbecco's modified Eagle's medium (k-o DMEM); and (3) induction of EBs generated in the k-o SRM to generate functional CMs by the addition of serum-containing media.

The normal culture medium supplemented by FBS is replaced by two different SRM, SRM2 or the k-o SRM, during cultivation of ES cells or incubation of EBs. For the ES cells to be adopted for these protocols, weaning of the cells cultured in medium supplemented with serum to medium supplemented with SRM is not generally required. In addition, we present the normal conventional protocol with serum-containing medium for culturing and differentiation of ES cells to CM as a standard reference protocol.

For in vitro differentiation studies, especially studies of signal cascades, SRM define more or less an ideal culture condition. Application of growth factors or hormones or inhibitors of growth factor receptors can be analyzed more accurately without the intervention by the serum because the composition of SRM is well defined and reproducibly made by the manufacturer. Therefore, there is no need to be concerned about potential experimental variations, unlike when serum-containing conditions are used.

The heart is the first functional organ of the embryo, and it arises as a beating and contracting area between day E6 and day E14 (postcoitum) during mammalian embryonic development. The contractility and beating is caused by CMs, which are derived from the embryonic mesoderm, the third germ layer. The mesoderm germ layer derives muscle cells, cartilage, bones, blood cells, vessels, and veins; the nervous system and skin are derived by the ectoderm, and the cavity organs, like the bladder and liver, are derived by the endoderm.

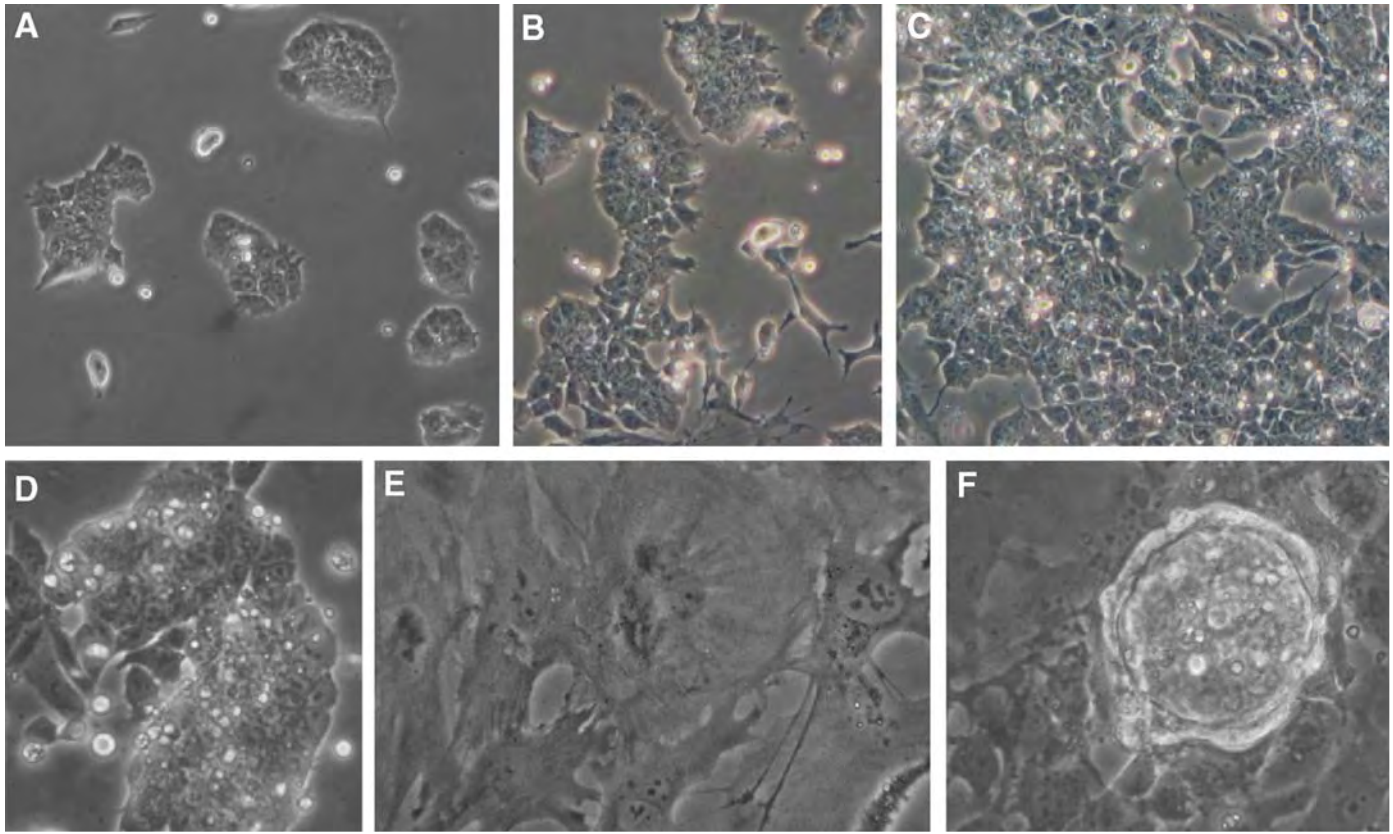

Fig. 1. *(Continued)*

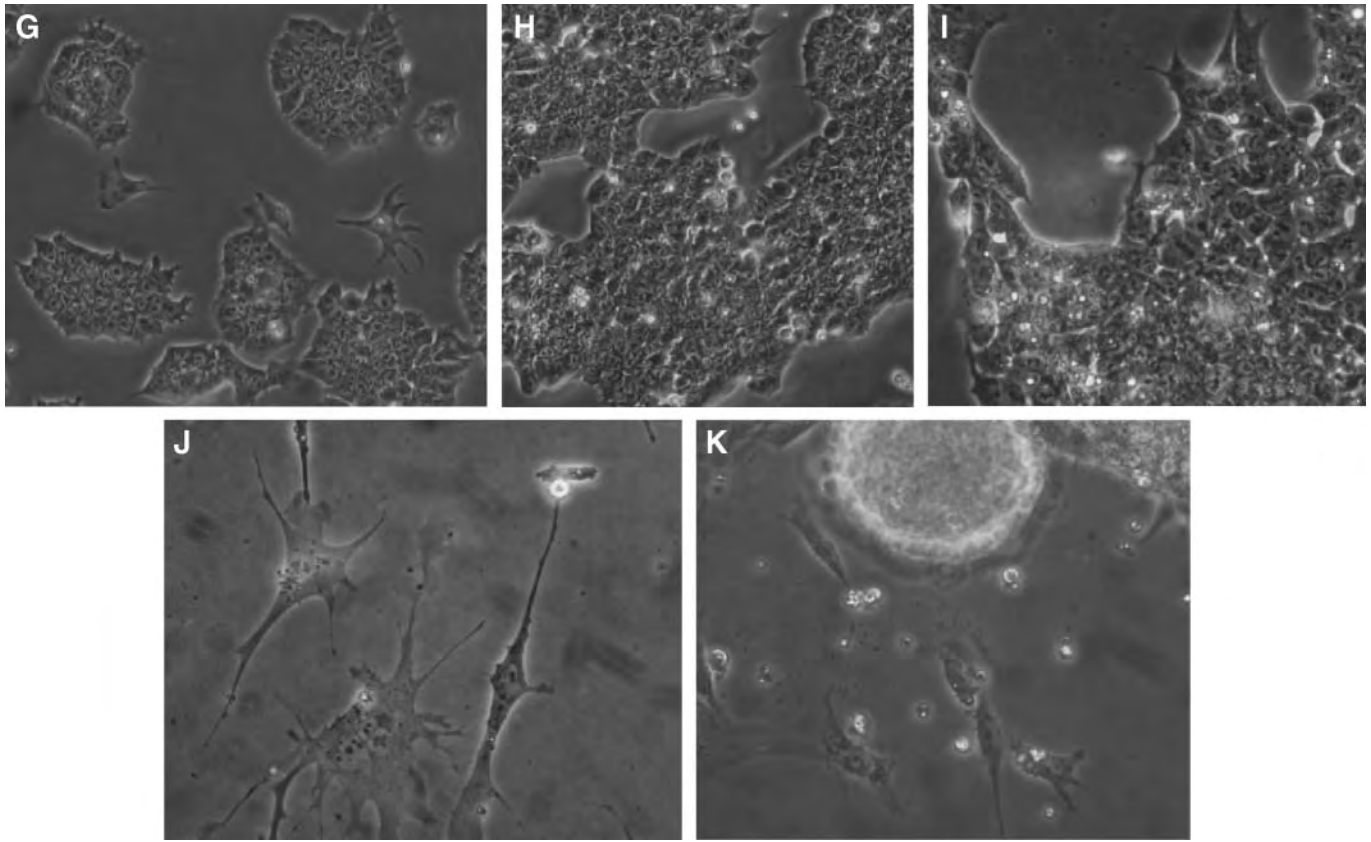

Fig. 1.

The differentiation of cardiogenic mesoderm requires interaction with the other germ layers, such as the embryonic endoderm and ectoderm as well as the extraembryonic endoderm ***(16)***. This interaction between the germ layers is facilitated by differential gene expression. The spatially and temporally regulated distribution of gene products, such as different hormones and growth factors and their receptors, transcription factors, and cell adhesion molecules, allows embryogenesis and the formation and function of the organs ***(17)***. Therefore, we analyzed the expression patterns of different transcription factors and hormones, such as the teratocarcinoma-derived growth factor cripto-1, primarily expressed in undifferentiated cells and later in the heart ***(18–20)***; the transcription factor GATA6, involved in heart formation and differentiation ***(21)***; the wingless-related MMTV integration site (wnt)-1, involved in the induction of the primitive streak and later in the heart differentiation ***(22,23)***; the T-box gene brachyury (T-bra), as a marker gene for the established and developing mesoderm ***(24–26)***; α-myosin heavy chain protein (α-MHC), as a marker for CMs; and the platelet-derived growth factor receptor β-type (PDGFR-β), involved in mesoderm differentiation ***(27)***. One of the essential components of serum is lipoproteins. Therefore, we analyzed the low-density lipoprotein receptor (LDLR), the very low-density lipoprotein receptor (VLDLR), and the apolipoprotein E receptor (Apo ER)-2. In addition, we analyzed the expression of the endothelial-differentiation gene 1 (EDG1) ***(28)*** and the platelet-derived cell adhesion molecule 1 (PECAM1) in ES cells and EBs for examining mesoderm differentiation into hematopoietic and endothelial tissue (for review, *see* **ref. *29***) to compare and contrast serum-free and conventional protocols (with serum) in terms of the differential gene expression profiles to prove the credibility of the protocols we established (**Fig. 3A**).

Fig. 1. Morphology of CGR8 embryonic stem (ES) cells in the presence and absence of LIF. (**A**) CGR8 ES cells cultured in ES cell culture medium supplemented with fetal bovine serum (FBS) and leukemia inhibitory factor (LIF) 24 h after thawing. The cells grow in small colonies consisting of small and tightly packed single cells. Vacuoles and nucleus appear to be dark bodies. (**B**) The same ES cell culture as shown in (**A**) 48 h after thawing. (**C**) The same ES cell culture as shown in **A** and **B** 3 d after thawing (confluency around 80%). The medium was changed 1 d before the picture was taken. This culture was used for the generation of embryoid bodies (EBs). (**D**) An overcrowded ES cell culture cultured in ES medium (confluency around 95%). This culture was not suitable for generation of EBs. (**E**) ES cells cultured for 10 d in culture medium supplemented by FBS in the absence of LIF. The morphology of the cells is significantly changed. Some of these differentiated cells show a neuronlike differentiated phenotype. (**F**) ES cells cultured for 19 d in culture medium supplemented by FBS in the absence of LIF. The bright spot is an EB-like structure. (**G**) CGR8 ES cells cultured in k-o ES cell culture medium supplemented with k-o SRM and LIF 24 h after passaging (passage number three under serum-free conditions). The cells grow in small colonies consisting of small and tightly packed single cells, exactly like the FBS-treated cells. (**H**) The same ES cell culture as shown in **G** 36 h after plating. (**I**) The same ES cell culture as shown in **G** 52 h after plating. (**J**) ES cells cultured 9 d in k-o ES cell culture medium supplemented by k-o SRM in the absence of LIF behave exactly the same as the ES cells cultured under serum conditions. (**K**) ES cells cultured 19 d in k-o culture medium supplemented by k-o SRM in the absence of LIF. The bright spot is an EB-like structure exactly the same as under serum conditions.

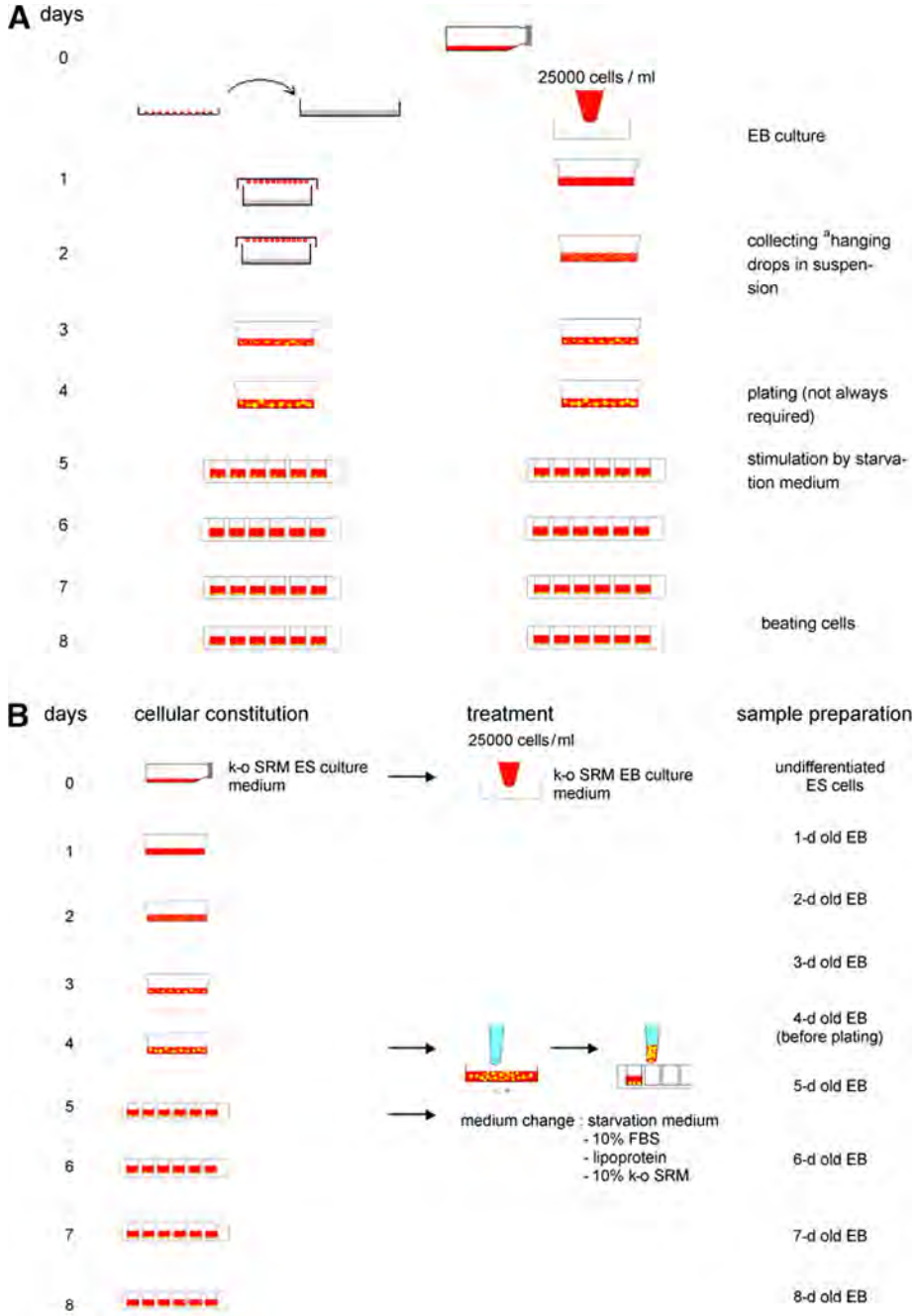

Fig. 2. *(Continued)*

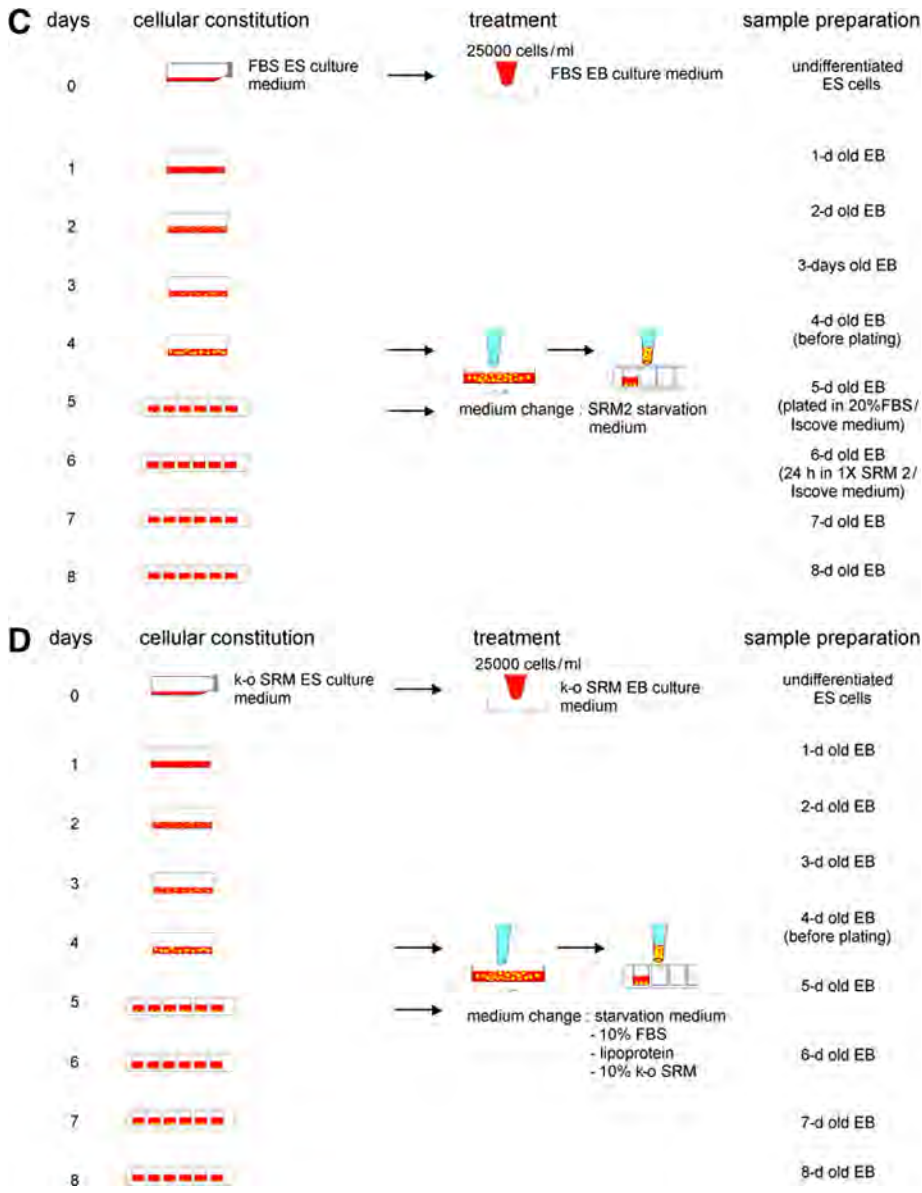

Fig. 2. *(Continued)*

We report here the above-mentioned three different serum-free ES culture protocols for the ES cell line CGR8 *(5)*. This cell line is derived from a male 3.5-d-old blastocyst-stage mouse embryo of mouse strain 129ola. These cells, unlike other ES cells, do not require mitotically inactivated fibroblasts, referred to as feeder cells, for their in vitro culture.

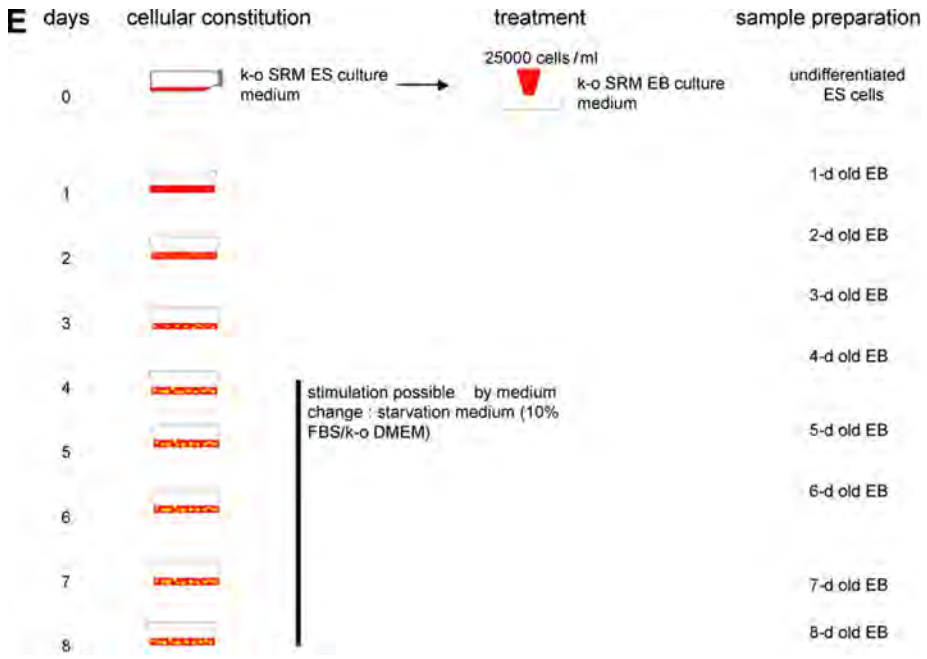

Fig. 2. (**A**) Generation and differentiation of the embryoid bodies (EBs). We used two different protocols for the generation of EBs. Both protocols required a stabilized embryonic stem (ES) cell culture, which was used for preparation of a 25,000 cells/mL cell suspension. Both protocols can be used for serum-free or serum-containing media. The hanging drop method is a variation of the most commonly used protocol for the generation of EBs. EBs were cultured for 4 d (or 7 d; data not shown) in the presence of high fetal bovine serum (FBS) concentration (20%) and plated at d 4 (or 7) in gelatin-coated dishes. The suspension method is entirely different from the dropping procedure. The cell suspension is directly placed in bacteriological dishes and incubated for 4 d. (**B**) Enhanced cardiac differentiation from plated EBs by the application of a reduced FBS concentration. ES cells were cultured in the presence of FBS, and EBs were generated in the presence of serum. The cardiac differentiation was specially enhanced by FBS starvation medium. The concentration of FBS was reduced from 20 to 0.2%. This starvation medium was added 24 h after plating, when the EBs collapse. (**C**) Induction of cardiac differentiation in plated EBs by SRM2. ES cells were cultured in the presence of FBS, and EBs were generated in the presence of serum. The cardiac differentiation was specially enhanced by serum replacement media 2 (SRM)-2 starvation medium. This starvation medium was applied 24 h after plating when the EBs collapse. (**D**) Induction of cardiac differentiation under serum-free conditions. ES cells and EBs were cultured serum-free in k-o medium. The cardiac differentiation was specially induced by FBS, k-o SRM, or lipoprotein starvation medium. This starvation medium was applied 24 h after plating. The variation not shown allowed application of the starvation medium parallel to plating (*see also* **Fig. 4A**). (**E**) Induction of cardiac differentiation under serum-free conditions in unplated EBs. ES cells and EBs were cultured serum free in k-o medium. Unplated EBs in bacteriological dishes were stimulated by FBS-containing starvation medium. The stimulation can happen at 4–14 d (*see also* **Fig. 4A**). (Please *see* the companion CD for the color versions of this figure.)

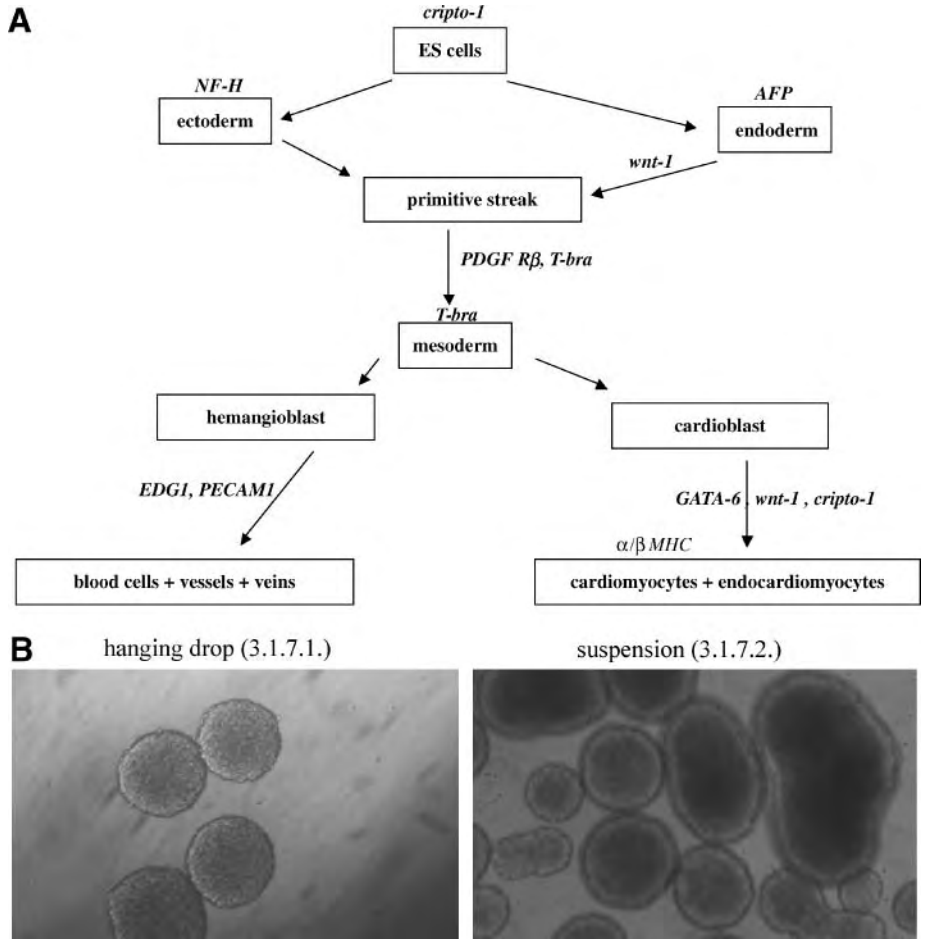

Fig. 3. *(Continued)*

Our investigations show that there are hardly any morphological differences between ES cell cultures and EBs cultured in serum-containing and serum-free medium (**Fig. 1**). The pattern of specific marker genes for different germ layers was the same for both serum-treated and serum-free cultured EBs (**Fig. 3D**). However, all SRM-treated EBs generated more and stronger beating cells than FBS-treated sister cultures (**Fig. 4A**,**B**). The cardiac differentiation is promoted in SRM2; the hematopoietic and endothelial differentiation is unaffected (**Fig. 3C**). Therefore, we conclude that the well-defined and the restricted supply of essential substances like growth factors and hormones by SRM leads to more specific differentiation and a reduced proliferation in our cultures and provides us an ideal platform to investigate the process of the CM differentiation from ES cells accurately and to characterize the CMs under ideal conditions.

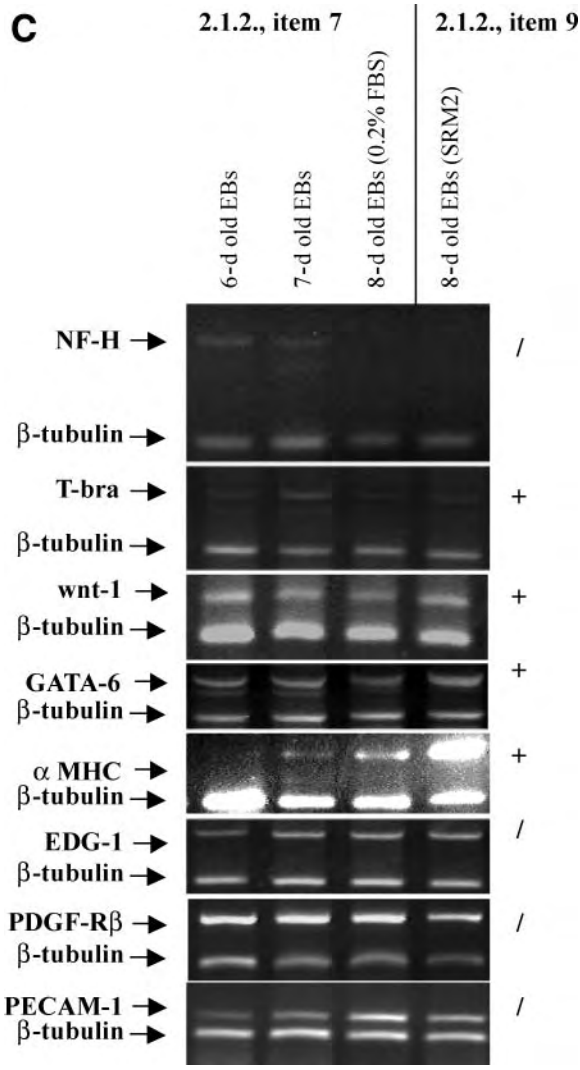

Fig. 3. *(Continued)*

1.1. ES Cell Culture and Generation of EBs

The preparation of EBs is described using ES cells derived from the CGR8 cell line. This cell line is distributed by the European Collection of Cell Cultures. The germline-competent cell line CGR8 was established from the inner cell mass of a 3.5-d-old male blastocyst-stage mouse embryo of the mouse strain *129ola*. This ES cell line does not need to be cultured on mitotically inactivated fibroblasts, also referred to as feeder cells. In the presence of LIF, ES cells remain undifferentiated, and they form a cellular layer consisting of small and tightly packed single cells. In the absence of LIF, ES cells

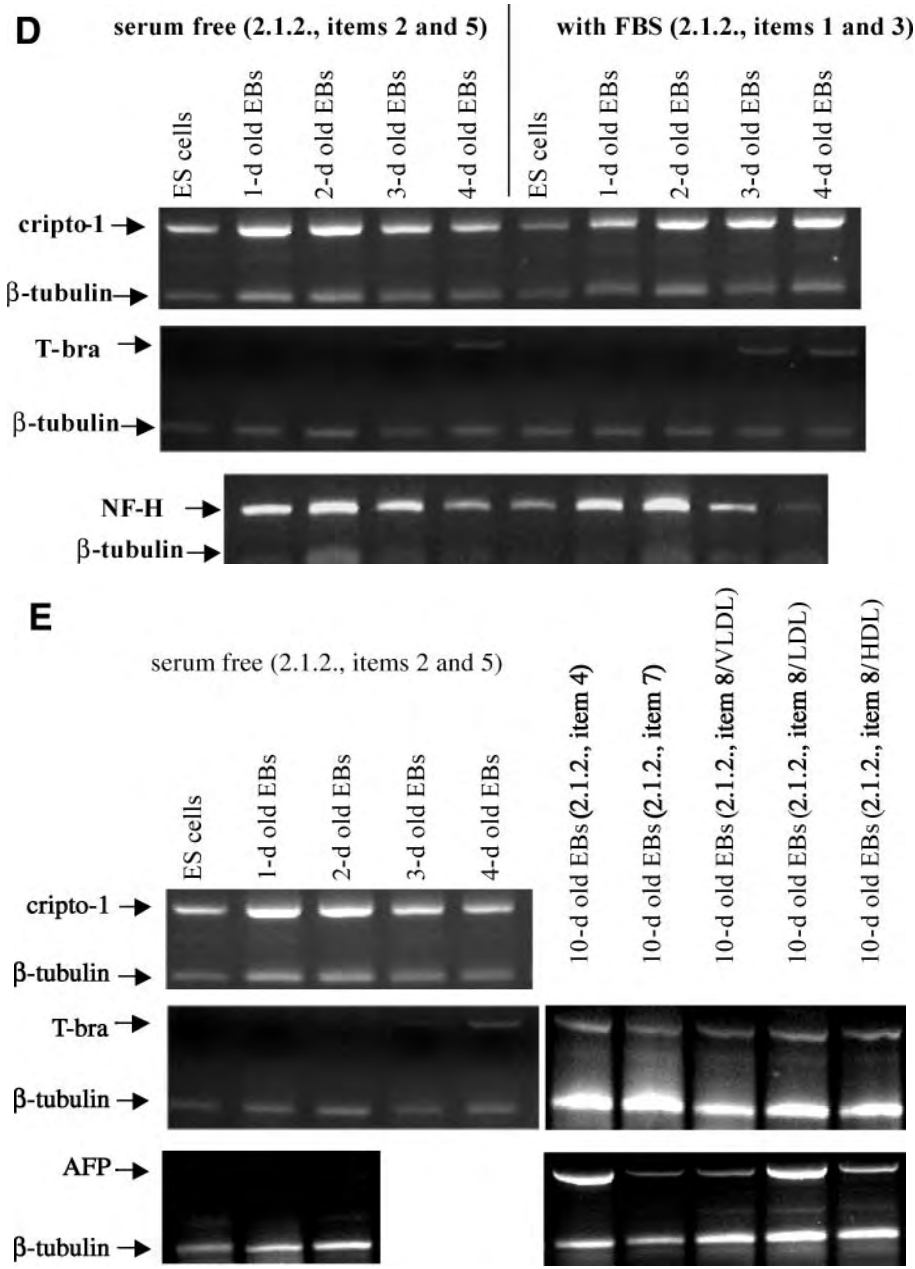

Fig. 3. *(Continued)*

differentiate spontaneously into different cell types (**Fig. 1**). Culture of CGR8 ES cells requires gelatin-coated cell culture flasks and LIF for propagation. After thawing, at least four passages are necessary for a stable ES cell culture for the generation of EBs.

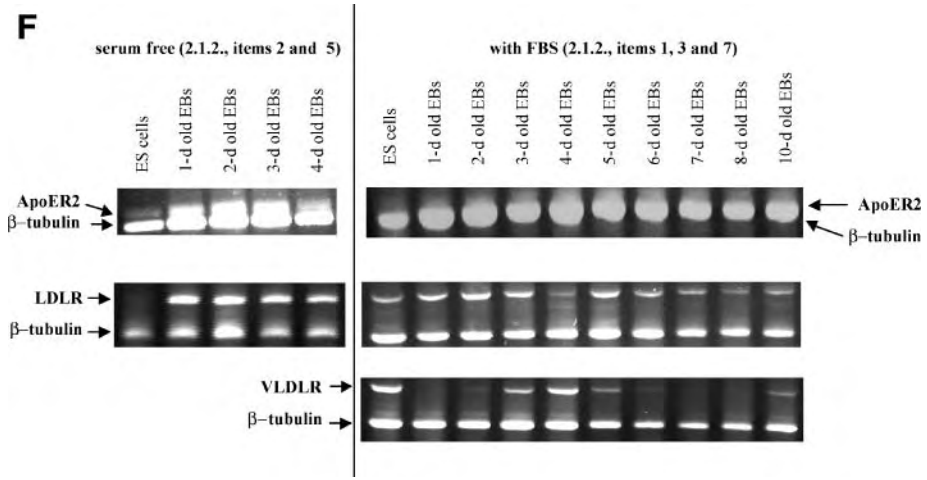

Fig. 3. (**A**) Gene expression profile during development. Genes are differentially expressed in a temporal and spatial manner during development. Some genes are involved in the induction of germ layers (shown next to the arrows) or in differentiated cell lineages (shown on top of the tissue boxes). (**B**) Morphology of embryoid bodies (EBs). EBs generated with the hanging drop and suspension protocols (**Fig. 2A**) differ in size and shape. EBs generated with the hanging drop procedure are rather homogeneous in shape and size; the EBs from the suspension protocol differ in size and shape. (**C**) Analysis of the low-serum (0.2% fetal bovine serum [FBS]) and serum-free (serum replacement media 2 [SRM]-2) EBs. We analyzed the expression pattern of the neurofilament heavy chain protein (NF-H) as an ectodermal marker; the T-box gene brachyury (T-bra) as a mesodermal marker; the wingless-related MMTV integration site 1 (wnt-1), which is involved in the induction of the primitive streak and connexin 43 expression in the heart; the GATA-transcription factor 6 (GATA6), which is involved in cardiac mesoderm differentiation; and α-myosin heavy chain protein (α-MHC), as a marker for beating CMs. The mesoderm generates endothelial and hematopoietic tissue. Therefore, we analyzed the endothelial differentiation gene 1 (EDG1) as a marker for endothelial differentiation; the platelet-derived growth factor β-type (PDGFR-β), which is involved in lateral plate differentiation; and the platelet endothelial cell adhesion molecule 1 (PECAM1) as markers for hematopoietic cells. The EBs were generated as described in **Fig. 2B** (0.2% FBS) and **Fig. 2C** (1X SRM2)./represents unaffected expression pattern; + indicates the upregulated expression patterns. All mesodermal and cardiac-specific genes were upregulated; the ectodermal NF-H or the endothelial and hematopoietic genes were unaffected in this serum-free condition. This correlates well with the observation that this culture condition produces more beating CMs compared to serum conditions. (**D**) Expression pattern in EBs generated under serum-free (k-o SRM) or serum-containing culture conditions. EBs 1–4 d old were generated using the suspension method and the serum-free k-o SRM protocol or the FBS-containing protocol. We analyzed the teratocarcinoma-derived growth factor 1 (cripto-1), the specific marker for undifferentiated ES cells and heart; brachyury (T-bra), as a mesodermal marker gene; and neurofilament heavy chain protein (NF-H), as a marker for the ectodermal germ layer. Significant difference between both types of EBs is not detected. (**E**) Expression pattern in EBs under serum-free conditions (k-o SRM) after stimulation by different starvation media. EBs were generated using the hanging drop method and the serum-free k-o SRM protocol. The EBs (plated at d 4) were stimulated by different starvation media at d 5. We used normal EB culture

In our laboratory, EBs are generated both by the hanging drop method and suspension method as illustrated in **Fig. 2A**. Briefly, a defined volume of an ES cell suspension is spotted in the form of a hanging drop in the lid of a Petri dish for 2 d. To allow further proliferation and to increase their size, EBs are then collected and transferred into bacteriological dishes with fresh media. For the suspension protocol, cells at a required density are seeded in suspension and incubated for 4 d in a bacteriological Petri dish. Finally, the EBs are plated on multiwell culture plates for their definitive differentiation. The serum-free culture method requires a very different passaging protocol to obtain subcultures. Our experience with this technique indicates that weaning of CGR8 ES cells or EBs to serum-free cell culture medium is not generally required.

2. Materials

2.1. Tissue Culture

1. CGR8 ES cell line, derived from a male 3.5-d old embryo from mouse strain 129ola (European Collection of Cell Cultures, Salisbury, Wiltshire, UK; cat. no. 95011018) (*see* **Note 1**).
2. T-25 tissue culture flasks (VWR, Langenfeld, Germany; Merck cat. no. 35014).
3. 10-cm Petri dishes (bacteriological) (Greiner, Solingen, Germany; cat. no. 664102).
4. 6-cm Petri dishes (bacteriological) (Greiner, cat. no. 628102).
5. Six-well cell culture plates (VWR, cat. no. 35-3046).
6. 24-well cell culture plates (VWR, cat. no. 35-3047).
7. 5-mL pipets (VWR, cat. no. 35-6543).
8. 10-mL pipets (VWR, cat. no. 35-6551).
9. 15-mL tubes (VWR, cat. no. 35-2097).
10. 50-mL tubes (VWR, cat. no. 35-2098).
11. Cryotubes (VWR, Nunc cat. no. 37-5418).
12. 0.2-µm sterile filter (VWR, Merck cat. no. 5167690).
13. Neubauer cell-counting chamber (VWR, cat. no. 631-1131).
14. Pasteur pipets (VWR, Merck cat. no. 612-1701).
15. Multistep pipet plus (Eppendorf, Hamburg, Germany; cat. no. 4981 000.019).

2.1.1. Commercial Media

1. 500 mL heat-inactivated, mycoplasma-tested FBS (Invitrogen, Karlsruhe, Germany; batch no. 40-F-4498K).
2. 100 mL SRM2 (50X; Sigma Aldrich, Taufkirchen, Germany; cat. no. S-9388).
3. 500 mL k-o SRM (Invitrogen, cat. no. 10828-028).

Fig. 3. *(Continued)* medium supplemented with 20% k-o SRM or 10% k-o SRM and the very low-density lipoprotein (VLDL), the low-density lipoprotein (LDL), the high-density lipoprotein (HDL), or FBS. The expression of the mesodermal marker gene *T-bra* was unaffected by the starvation media; the expression of the endodermal marker gene α-fetoprotein (AFP) was significantly upregulated under serum-free conditions or after application of LDL and VLDL. (**F**) Lipoprotein receptor expression under serum-free (k-o SRM) and serum-containing (FBS) culture conditions. EBs were generated using the suspension method and the serum-free k-o SRM protocol or the FBS-containing protocol. The lipoprotein receptors apolipoprotein receptor E2 (ApoER2), the low-density lipoprotein receptor (LDLR), and the very low-density lipoprotein receptor (VLDLR) were expressed in both culture conditions in the same manner. Significant differences between their expression profiles are not seen.

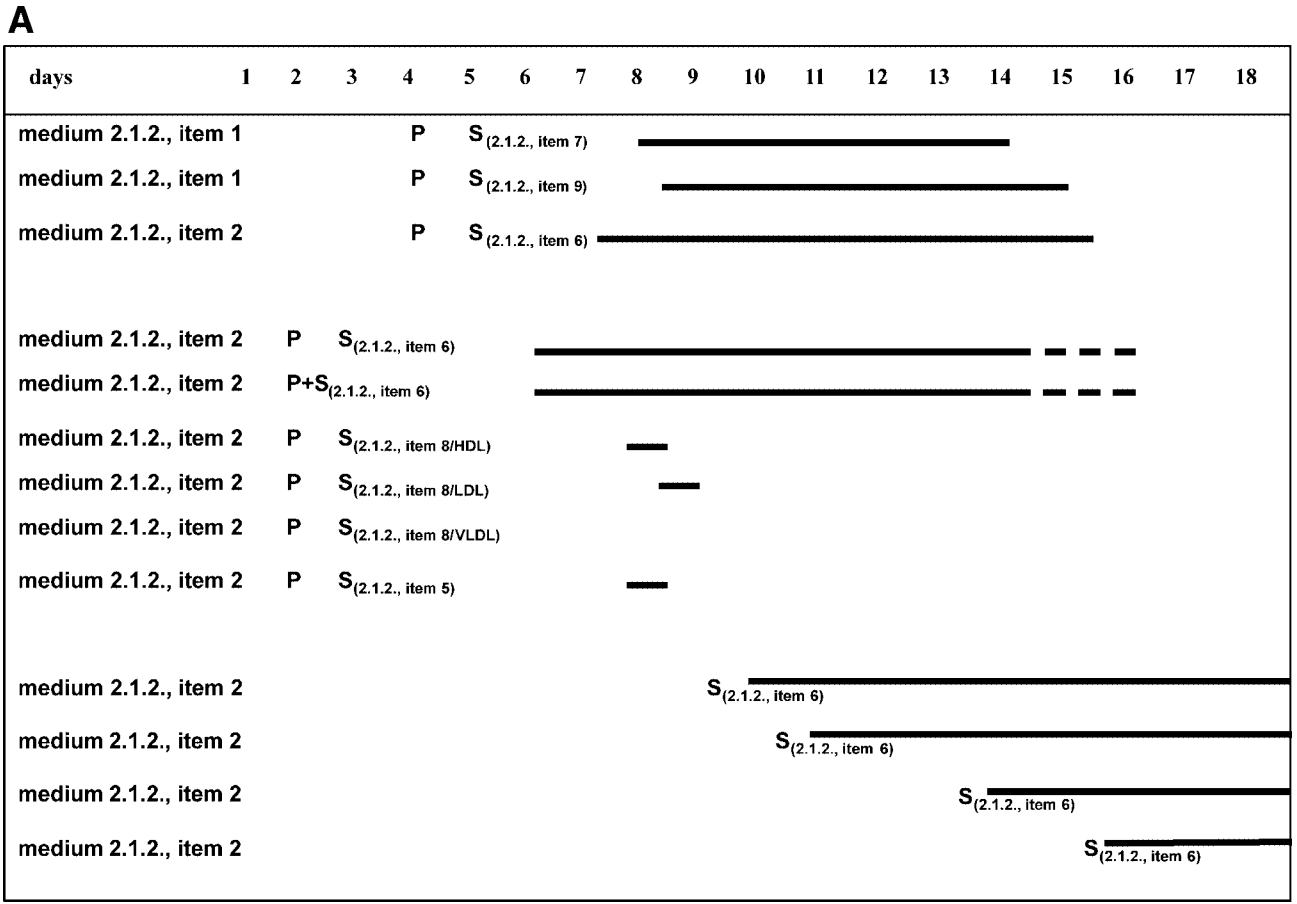

Fig. 4. *(Continued)*

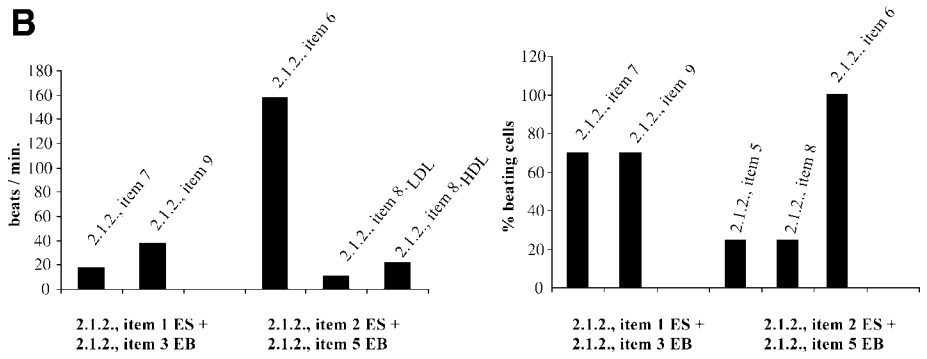

Fig. 4. (**A**) Cardiac differentiation in CGR8 embryoid bodies (EBs) using different protocols. EBs were generated and cultured in the presence or absence of fetal bovine serum using the general method. Cardiogenesis was induced by different starvation media (**S**) in plated (**P**) or unplated EBs. The solid bars show when the cells had cardiac contractions. Only the serum-free starvation medium supplemented by the very low-density lipoprotein had not produced cardiomyocytes (CMs). (**B**) Number of beats per minute in CMs induced by different starvation media. The number of beats (contractions) per min of several beating areas was counted for different starvation media used. (**C**) Percentage of beating CM population against total number of EBs plated. The total number of wells included per assay ranged from four to eight.

4. 500 mL buffered Glasgow minimal essential medium (GMEM) (Sigma Aldrich, cat. no. G-5154).
5. 500 mL Iscove's modified Dulbecco's medium (IMDM) (Invitrogen, cat. no. 21980-065).
6. 500 mL knockout Dulbecco's modified Eagle's medium (k-o DMEM) (Invitrogen, cat. no. 10829-018).
7. 500 mL phosphate-buffered saline (PBS) without calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions (w/o Ca^{2+}/Mg^{2+}) (Invitrogen, cat. no. 14190-094).
8. 100 mL trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Ca^{2+}/Mg^{2+} free), stored at –20°C (Invitrogen, cat. no. 25300-054).
9. PBS*: 500 mL PBS with Ca^{2+} and Mg^{2+} (Invitrogen, cat. no. 14040-091).
10. LIF (1000 U/μL concentrated) (Sigma Aldrich, cat. no. L-5158).
11. 100 mL 200 m*M* L-glutamine (100X; Invitrogen, cat. no. 25030-024) (*see* **Note 2**).
12 β-Mercaptoethanol (14.3 *M*; Sigma Aldrich, cat. no. M-7522). Prepare 0.1 *M* β-mercapto ethanol solution by dilution of 14.3 *M* β-mercaptoethanol: dilute the stock solution 1:143 with PBS to obtain 0.1 *M* β-mercaptoethanol. Store in the dark at 4°C for a maximum of 5 d (*see* **Note 3**).
13. Gelatin type II (Sigma Aldrich, cat. no. G-2500): gelatin solution is prepared as fivefold concentrated stock solution. Solubilize 5 g type II gelatin in 500 mL PBS and incubate for 30 min at 37°C. Autoclave the 1% gelatin solution twice for 30 min. To coat plates, prepare a 0.2% gelatin solution: dilute the 1% gelatin solution 1:5 with PBS.
14. 500 mL dimethyl sulfoxide (DMSO) (Sigma Aldrich, cat. no. D-8418).
15. Adenosine (Sigma Aldrich, cat. no. A-4036).
16. Guanosine (Sigma Aldrich, cat. no. G-6254).
17. Cytidine (Sigma Aldrich, cat. no. C-4654).
18. Thymidine (Sigma Aldrich, cat. no. T-1895).

19. Uridine (Sigma Aldrich, cat. no. U-3003).
20. Nucleotide solution: dissolve nucleotides (adenosine, cytosine, guanosine, uridine, and thymidine) in 100 mL double distilled water and incubate at 37°C for 30 min. Sterilize through a 0.2-µm filter and store it at 4°C. The final medium concentration should be 80 mg adenosine, 85 mg guanosine, 73 mg cytidine, 24 mg thymidine, and 73 mg uridine (*see* **Note 4**).
21. Sodium bicarbonate (7.5% w/v) (Invitrogen, cat. no. 25080-060).
22. 100 mL minimum essential medium (MEM) nonessential amino acids (100X; Invitrogen, cat. no. 11140-035).
23. 2.5 L ethanol (98% absolute; VWR, Merck cat. no. 8.18760.2500).

2.1.2. Media (see ***Tables 1*** *and* ***2*** *for details)*

1. ES cell culture medium: CGR8 ES cells are maintained in GMEM supplemented with 10% heat-inactivated FBS, 2 m*M* L-glutamine, 50 µ*M* β-mercaptoethanol, and 100 U/mL LIF (*see* **Note 5**). To prepare 10 mL ES cell culture medium, combine 8.9 mL GMEM, 1 mL FBS, 100 µL 200 m*M* L-glutamine, 1 µL 1000 U/µL LIF, and 5 µL 0.1 *M* β-mercaptoethanol.
2. Supplemented k-o DMEM: CGR8 ES cells are maintained in k-o DMEM (479.4 mL) supplemented with 5 mL nonessential amino acids, 9.6 mL 7.5% sodium bicarbonate, and 6 mL nucleotide solution ***(30)***. Shortly before use, add 10% k-o SRM, 2 m*M* L-glutamine, 50 µ*M* β-mercaptoethanol, and 100 U/mL LIF. To prepare 10 mL culture medium, combine 8.9 mL supplemented k-o DMEM, 1 mL k-o SRM, 100 µL 200 m*M* L-glutamine, 1 µL 1000 U/µL LIF, and 5 µL 0.1 *M* β-mercaptoethanol. Store the medium at 4°C and use within the next 5 d.
3. EB cultivation medium: supplement IMDM with 100 µ*M* β-mercaptoethanol, 2 m*M* L-glutamine, and 1X concentrated MEM. To prepare 500 mL supplemented IMDM, combine 490 mL IMDM, 5 mL 200 m*M* L-glutamine, 5 mL 100X MEM, and 3.5 µL 14.3 *M* β-mercaptoethanol. Use within 5 d. Prepare the final serum-containing EB cultivation medium by adding 20% FBS. For example, combine 80 mL supplemented IMDM and 20 mL FBS.
4. IMEM k-o SRM medium: combine 40 mL supplemented IMDM with 10 mL k-o SRM.
5. Serum-free k-o EB cultivation medium. Prepare supplemented k-o DMEM and add 20% k-o SRM, 100 µ*M* β-mercaptoethanol, and 2 m*M* L-glutamine. To prepare 100 mL, combine 79 mL supplemented k-o DMEM medium, 20 mL k-o SRM, 1 mL L-glutamine, and 100 µL 0.1 *M* β-mercaptoethanol (*see* **Note 6**).
6. k-o starvation medium: mix supplemented k-o DMEM, 100 µ*M* β-mercaptoethanol, 2 m*M* L-glutamine, and 10% FBS. For 50 mL, combine 44.5 mL supplemented k-o DMEM with 50 µL 0.1 *M* β-mercaptoethanol, 0.5 mL L-glutamine, and 5 mL FBS.
7. Iscove starvation medium: mix supplemented IMDM with 0.2% FBS. For 50 mL, combine 49.9 mL supplemented IMDM and 0.1 mL FBS.
8. Lipoprotein starvation medium: extract lipoprotein from human blood as described in **ref.** ***31***. Measure the lipoprotein concentration and use a final concentration of 100 µg/mL. Mix supplemented k-o DMEM with 100 µ*M* β-mercaptoethanol, 2 m*M* L-glutamine, 10% k-o SRM, and 100 µg/mL lipoprotein finally. For 50 mL, combine 44.5 mL supplemented k-o DMEM, 50 µL 0.1 *M* β-mercaptoethanol, 0.5 mL L-glutamine, 5 mL k-o SRM, and the defined amount of lipoprotein.
9. Iscove starvation medium with SRM2: mix supplemented IMDM and 1X diluted SRM2. For 50 mL, combine 49 mL supplemented IMDM with 1 mL 50X concentrated SRM2.
10. Cryoconservation medium for freezing undifferentiated ES cells: mix FBS (90%) with 10% DMSO. For 10 mL, combine 9 mL FBS with 1 mL DMSO. Keep this medium in the dark or prepare it fresh before use, corresponding to the lifetime of DMSO. This medium should be placed on ice for 30 min before use.

Table 1
Cell Culture Media Composition

	ES cell culture	Serum-free ES cell culture	Supplemented IMDM	Supplemented k-o DMEM
Medium	GMEM	Supplemented k-o DMEM	IMDM	k-o DMEM
LIF	100 units/mL	100 units/mL	—	—
β-Mercaptoethanol	50 μ*M*	50 μ*M*	100 μ*M*	—
L-Glutamine	2 m*M*	2 m*M*	2 m*M*	—
MEM	—	—	One-fold	One-fold
FBS	10%	—	—	—
SRM2	—	—	—	—
k-o SRM	—	10%	—	—
Sodium bicarbonate 7.5%	—	—	—	9.6 mL/500 mL
Nucleotide (adenine, cytosine, etc.) solution	—	—	—	6 mL/500 mL

GMEM or IMDM was used for generation of hanging drop EBs or k-o DMEM for serum-free conditions. IMDM is HEPES buffered, and k-o DMEM is phosphate buffered. GMEM is carbonate buffered and not qualified for the hanging drop method.

Table 2
EB Media Composition

	EB culture	EB culture	FBS starvation medium	SRM2 starvation medium
Medium	Supplemented IMDM	Supplemented IMDM	Supplemented IMDM	Supplemented IMDM
FBS	20%	—	0.2%	—
SRM2	—	—	—	Onefold
k-o SRM	—	20%	—	—
	EB culture	EB culture	EB culture	
Medium	Supplemented k-o SRM	Supplemented k-o SRM	Supplemented k-o SRM	
β-Mercaptoethanol	100 μ*M*	100 μ*M*	100 μ*M*	
L-Glutamine	2 m*M*	2 m*M*	2 m*M*	
FBS	—	10%	—	
SRM2	—	—	—	
k-o SRM	20%	—	10% + 100 μg/mL HDL, LDL, or VLDL	

HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein.

11. Wash medium supplemented with FBS: prepare a special 5% FBS containing wash medium. For 50 mL, combine 47.5 mL GMEM or IMDM with 2.5 mL FBS (*see* **Note 7**).
12. Serum-free wash medium: prepare serum-free wash medium with k-o DMEM supplemented with 5% k-o SRM: combine 47.5 mL k-o DMEM from the manufacturer's bottle with 2.5 mL k-o SRM.

2.1.3. General Comments Regarding Tissue Culture

1. Standard conditions for the incubation of mammalian cells require 37°C in a humid atmosphere with 5% carbon dioxide (CO_2) (*see* **Note 8**).
2. To prevent contamination by bacteria and fungi, use only sterile cell culture plastic materials and media for cultivation and media preparation. Autoclave all self-prepared solutions or sterilize by filter. Fungi are highly resistant; therefore, avoid using glasswares such as bottles or glass pipets.
3. Flame Pasteur pipets inside the lamina flow cabinet before using and the lids from opened bottles before closing.
4. Before placing something inside the lamina flow cabinet, clean the outer surface with 70% ethanol.
5. The use of antibiotics like penicillin or streptomycin is inadvisable. In our experiments, penicillin-streptomycin-treated ES cell cultures were unable to differentiate as usual and did not generate beating cells. Furthermore, microcontaminations will not be visible in the presence of the antibiotics.
6. Store the media according to the manufacturer's instructions or at least at 4°C and warm all solutions to 37°C before use.

2.1.4. Tissue Culture Equipment

1. Humidified incubator at 37°C and 5% CO_2.
2. Laminar flow cabinet.
3. 37°C water bath (*see* **Note 9**).
4. Central-CL2 centrifuge (Thermo Electron GmbH, Dreieich, Germany).
5. Inverted microscope with a range of phase contrast objectives (×10, ×20, ×32) equipped with photographic capabilities.
6. Liquid nitrogen tank.
7. Pipetmen (1, 2, 5, 10, and 25 mL).
8. Refrigerator (4°C), freezer (–20°C), and deep freezer (–80°C).

2.2. Molecular and Gene Expression Analysis

1. *Taq* DNA polymerase (5 U/µL; Promega, Mannheim, Germany; cat. no. M-1665).
2. Deoxyribonucleoside triphosphates (dNTPs) (100 µmol per NTP) (Promega, cat. no. U 1240).
3. Oligo-dT (50 pmol/µL; Promega, cat. no. C1101).
4. Random primers (50 pmol/µL; Promega, cat. no. C1181).
5. ImProm II kit (Promega, cat. no. A3800).
6. RNasin (Promega, cat. no. N2111).
7. ImProm II reverse transcriptase with 5X concentrated buffer (Promega, cat. no. A3802).
8. Tri-Reagent kit (200-mL bottle; Sigma Aldrich, cat. no. T-9424).
9. 100-bp ladder length standard (Promega, cat. no. G2101).
10. Chloroform.
11. Isopropanol.
12. Tris-HCl.
13. Boric acid.
14. EDTA: prepare 0.5 L 0.5 *M* EDTA at pH 8.0: dissolve 93.05 g EDTA in 500 mL H_2O. Adjust to pH 8.0 with NaOH and autoclave for 20 min.
15. Sodium hydroxide (NaOH) tablets.
16. Diethylpyrocarbonate (DEPC).
17. Low-melting agarose.

18. Ethidium bromide: dissolve 1 g ethidium bromide in 10 mL autoclaved double-distilled water, corresponding to a final concentration of 100 mg/mL. Dilute this solution 1:10, corresponding to a final concentration of 10 mg/mL. Store the solutions in the dark.
19. 0.5-mL soft (thin wall) tubes for polymerase chain reaction (PCR) (Biozym Diagnostic GmbH, Oldendorf, Germany; cat. no. 710910).
20. 0.5-, 1.5-, and 2-mL safe-lock normal reaction tubes (VWR).
21. DEPC H_2O: combine 0.01% DEPC with double-distilled water. Incubate at room temperature overnight with gentle shaking. Autoclave twice for 20 min (*see* **Note 10**).
22. 1X TBE buffer: dissolve 89 m*M* Tris-HCl, 89 m*M* boric acid, and 2 m*M* EDTA at pH 8.0 in double-distilled water.
23. Agarose gel: melt 2 g low-melting agarose in 100 mL 1X TBE in a microwave. Cool the solution to 60°C, add 10 µL 10 mg/mL ethidium bromide, and place it into a gel tray.
24. Synthetic oligonucleotides (PCR primers; *see* **Table 3**). The oligonucleotides were selected by *Oligo primer analysis software* (version 6.6.3.) (Med Probe, Oslo, Norway). We have cross-checked all primer pairs by the NCBI BLAST. Dissolve lyophilisates in 10 m*M* Tris-HCl at pH 7.0 to obtain a 50 pmol/µL solution.
25. PCR thermocycler with hot bonnet.
26. Tabletop centrifuge.
27. Gel apparatus.
28. Ultraviolet (UV) light source (UV lamp).
29. pH measurement equipment.
30. Micromolecular pipets and tips (20, 200, and 1000 µL).

3. Methods

3.1. Tissue Culture

3.1.1. Coating Gelatin Plates

1. Dispense 5 mL or 4 mL 0.2% gelatin solution, respectively, into either T-25 cell culture flasks or each well of a six-well plate.
2. Incubate gelatin-coated flasks or plates for at least 3 h (or overnight) in a sterile humidified atmosphere.
3. Aspirate off the gelatin solution shortly before use and prevent drying of gelatin-coated dishes or plates.

3.1.2. Thawing of ES cells (see **Notes 11** and **12**)

1. Fill a 50-mL tube with 30 mL GMEM wash medium warmed to 37°C.
2. Remove a frozen vial from liquid nitrogen (*see* **Note 13**) and place it immediately into a 37°C water bath for 2 min (*see* **Note 14**).
3. Slowly add some wash medium to the vial and transfer the entire suspension into the 50-mL tube containing the pre-prepared wash medium.
4. Spin down for 5 min at 140*g* at room temperature.
5. Aspirate the medium and resuspend the cell pellet in 5 mL ES cell culture medium, gently pipetting up and down to obtain a single-cell suspension.
6. Aspirate the gelatin solution from a flask and transfer the cell suspension from the 50-mL tube into the gelatin-coated flask.
7. Place the dish in the incubator at 37°C and 5% CO_2 and incubate overnight.
8. Examine the cellular morphology and check for contamination each day. Change the medium after 24 h to remove the dead cells and then change it every other day thereafter.

Table 3
Oligonucleotide Primers for RT-PCR Analysis

Gene	Forward primer	Forward primer	Size (bp)
Teratocarcinoma-derived growth factor (Cripto-1) (M87321)	TAGCCTTGGGTGTTTCGAGA	CAGTGCTCTTTGCGAACATCA	395
Neurofilament heavy chain (NF-H) (M35131)	GCCCAAGAGGAGATAACTGA	TTCTGTCACTCCTTCCGTCAC	477
α-Fetoprotein (AFP) (V00743)	CCAGAACCTGCCGAGAGTTGC	GCCTTCAGGTTTGACGCCATT	552
T-box gene brachyury (T-bra) (NM_009309)	GCTGTTGGGTAGGGAGTCAAG	AAATTGGGCGAGTCTGG	446
Wingless-related MMTV integration site 1 (wnt-1) (K02593)	GCCGAGAAACAGCGTTCATC	GGTTCATGAGGAAGCGTAGG	236
GATA transcription factor 6 (GATA6) (AF179425)	GACCTGGAGGTGCGGGATCGG	CAGCGGTGCGGCCATGTAGGG	281
α-Myosin heavy chain (α-MHC) (M76599)	AAGCGCAATGCAGAGTCGGT	GTATTGGCCACAGCGAGGGTC	354
Platelet-derived growth factor β-type (PDGF-Rβ) (BC055311)	CGTCTCAGTGACATCCGTGAC	GAGTGATACCAGCTTTAGTCC	398
(BC055311)Endothelial differentiation gene-1 (EDG1) (NM_007901)	AATCTTAGATGATACGGGAAA	ATACCTAGTGACAGCCCGAAAT	418
Platelet / endothelial cell adhesion molecule 1 (PECAM1) (AF412280)	CACATCTGCAATCTCGGGTGT	GAAGAGGCGATTGTCACGTTC	268
Low-density lipoprotein (LDL) (BC 019207)	GACGGCTCCGATGAGTGGC	CACATTGACGCAGCCGAGCTC	299
Very low-density lipoprotein(VLDL) (NM_013703)	CGGTGGCTCTCGATGCGGACA	CCAGTAAACAAAGCCCGACA	310
Apolipoprotein receptor E 2 (ApoER) (AJ312058)	CCTGCCGAGAAGTTAAGC	CCGCTCCTGGTTGCAC	210
β-Tubulin	AATGGGCACTCTCCTTATCAG	GGCCTCGTTGTCAATGCAATA	181

3.1.3. Freezing ES Cells

1. Prepare cryopreservation medium and incubate for 30 min on ice.
2. Label a cryotube with all-important data (date, passage, cell line) and place it on ice.
3. Aspirate the medium from a confluent ES cell culture dish and continue to harvest the cells following the protocol up to **step 9** in **Subheading 3.1.4.**
4. Remove the supernatant.
5. Rock the tube to loosen the cell pellet and add a defined volume of ice-cold cryoconservation medium to obtain a suspension with 1×10^6 cells/mL (*see* **Note 15**).
6. Fill 1.8 mL of this cell suspension into each cryotube (*see* **Note 16**) and close it.
7. Store the cryotube for the next 48 h in a freezer at –80°C.
8. Next place the cryotube into liquid nitrogen and store until use.

3.1.4. Subculturing ("Passaging") of ES Cell Cultures

ES cells are highly sensitive; cell contact can inhibit the cellular growth and leads to differentiation. Therefore, passage or subculture ES cells strictly when the culture reaches around 70% confluence (*see* **Note 17**).

1. Aspirate the medium and wash off the ES cell culture twice with 37°C PBS.
2. Aspirate the PBS and add 2–3 mL room temperature trypsin-EDTA solution. Incubate at 37°C for 3–5 min until the cells have lost their adhesion to the flask and with each other. Monitor the reaction under the microscope and rock the flask a little to destroy cellular aggregates mechanically (*see* **Note 18**).
3. Add 5–7 mL wash medium (with or without serum) and transfer the suspension into a 15-mL tube (*see* **Note 19**).
4. Pipet the suspension up and down several times to generate a single-cell suspension. Monitor under the microscope to be sure that there are no clumps.
5. Spin down for 15 min at 140*g* at room temperature.
6. Aspirate the supernatant and resuspend the cells in 5 mL wash medium (with or without serum).
7. Take a small (20-µL) aliquot for the viable cell count.
8. Spin down for 15 min at 140*g* and room temperature.
9. Estimate the cell titer in suspension using a cell-counting chamber. Prepare the cell-counting chamber as described by the manufacturer. Add the appropriate volume of cell suspension. Count the cells under a microscope and calculate the total cell number in your cell suspension as described (*see* **Note 20**).
10. Aspirate the supernatant and resuspend the cells in the calculated volume of ES cell culture medium (with and without serum) to obtain a single-cell suspension of 1×10^6 cells/mL.
11. Aspirate the solution from a gelatin-coated flask or one well of a six-well plate and fill it with 4.5 mL or 3.7 mL ES cell culture medium, respectively.
12. Seed the flask or the six-well plate well with 0.5 mL or 0.3 mL of the 1×10^6 cells/mL ES cell suspension, corresponding to 5×10^5 or 3×10^5 ES cells, respectively.
13. Change the medium after 24 h or passage the cells after 2 d in the case that they are confluent (*see* **Note 21**).

3.1.5. Switching ES Cells to Serum-Free Conditions

1. Thaw ES cells as described in **Subheading 3.1.2.**
2. When the cells reach 30% confluency, replace the existing serum-containing medium with k-o SRM containing serum-free ES cell culture medium.

3. Hereafter, culture the cells in serum-free ES cell culture medium until they reach 60–70% confluency and then subculture as described in **Subheading 3.1.6.**

3.1.6. Passaging ES Cells Under Serum-Free Conditions

1. Subculture ES cells as described in **Subheading 3.1.4.** using both serum-free wash medium and culture medium.
2. Reduce the incubation time after adding the trypsin-EDTA solution (*see* **Note 22**).
3. Dilute Trypsin with PBS* or serum-free washing medium.
4. Passage cells as in **Subheading 3.1.4.** when cultures reach 60–70% confluence using k-o SRM containing serum-free ES cell culture medium (*see* **Note 23**).

3.1.7. Preparation of EBs

The generation of EBs requires a 70–80% confluent ES cell culture. Use only ES cells cultured at least for four or five passages after the thawing procedure. At this stage only, the cells will have exponential growth with stabilized properties and will generate right-size, round EBs (**Fig. 3B**). For generation of serum-free EBs, use serum-free cultured ES cells (passaged at least three times in serum-free conditions). The procedure for the generation of EBs is the same in both serum-free and serum-containing conditions. However, the serum-free EB culture medium described in **Subheading 2.1.2.**, **item 5**, is buffered by carbonate (Dulbecco's medium), and Iscove's medium is buffered by HEPES. Therefore, IMDM-containing media (**Subheading 2.1.2.**, **items 3** and **4**) are more suitable for generation of hanging drops.

EBs can be generated by two different methods (*see* **Notes 24** and **25**): hanging drop and suspension.

3.1.7.1. The Hanging Drop Method

The EBs generated via the hanging drop method will be well defined and highly similar in shape and size. This method, however, is labor intensive and rather tedious. Also, each hanging drop will likely not generate an EB; therefore, multiple hanging drops should be prepared to ensure ample EBs are generated.

1. Choose 65–70% confluent ES cells at least 4–11 passages after thawing.
2. Trypsinize ES cells as described in **Subheading 3.1.4.** up to **step 9** or in **Subheading 3.1.6.**
3. Resuspend the pellet in EB culture medium (**Subheading 2.1.2.**, **items 3**, **4**, or **5**) to obtain a 1×10^6 cells/mL cell suspension.
4. Dilute this suspension with EB culture medium to obtain 2.5×10^4 cells/mL (1:40).
5. Fill 30 10-cm Petri dishes with 7 mL PBS each.
6. Pipet the cell suspension gently up and down to obtain a homogenized single-cell suspension. Repeat this before filling up the multistep pipet.
7. Use the multistep pipet to drop 20 µL/drop of this cell suspension onto the lid of the bacteriological Petri dish, placing about 60–70 drops/lid, keeping 0.5 cm between the drops and 1 cm from the edge.
8. Carefully invert the lid with drops and close the Petri dish. Incubate the hanging drops for 2 d.
9. Open the dish after 2 d in a sterile atmosphere.
10. Use a 5-mL pipet and 3 mL EB culture medium to collect the drops from the lid.
11. Collect the EBs from three lids in one new 10-cm Petri dish with 12 mL EB culture medium.
12. Incubate for another 2–5 d at 37°C and 5% CO_2.

3.1.7.2. The Suspension Method

The suspension method will generate EB cultures. The number of EBs is very high, but the EBs often differ in size and shape. Therefore, you have to select right-size EBs from the whole culture for subsequent assays.

1. Follow the protocol up to **step 4** as described in **Subheading 3.1.7.1.**
2. Pipet the cell suspension up and down to obtain a homogenized cell suspension.
3. Fill 5 mL of the ES cell suspension into a 6-cm bacteriological Petri dish.
4. Close the Petri dish and incubate the suspension for 4–7 d at 37°C and 5% CO_2.

3.2. CM Generation Via the Three Different Serum-Free Culture Protocols

EBs can be cultured serum free during generation or during the later differentiation time. Because the detection of CMs is easier in plated EBs, plate 4-d-old EBs/7-d-old EBs in 0.2% gelatin-coated dishes. The spheroidal structure of the EBs collapses 24 h after plating. As an example, a plated EB is shown in **Fig. 5C**. The generated EBs can be serum-free cultured after plating by medium change. Beating cells will first be detectable in 7- to 9-d-old EBs.

3.2.1. Standard Version of CM Generation (Hunger Protocol)

In the standard version of CM generation (the hunger protocol), EBs are generated and cultured in medium supplemented with FBS. The FBS concentration is reduced to 0.2% after plating.

1. Prepare CGR8 EBs as described in **Subheading 3.1.7.1.** or **3.1.7.2.** using the FBS-supplemented EB culture medium.
2. Aspirate the gelatin solution from a 24-well plate and fill each well with 0.5 mL EB culture medium.
3. Use a blue tip and the micromolecular pipet to pick the EBs from the suspension and transfer 20 EBs into each well counting under the microscope (*see* **Note 26**).
4. Incubate the plated EBs overnight at 37°C and 5% CO_2 in EB culture medium.
5. Change the medium into EB starvation medium 24 h after plating.
6. Incubate the plates at 37°C and 5% CO_2 and check for beating areas and changes in their cellular morphology under the microscope in subsequent days.
7. Change the medium every other day.
8. Beating cells can be monitored and documented by photography or videography.

3.2.2. The First Variation of the Standard Differentiation Protocol

The first variation of the standard differentiation protocol requires EBs generated in the presence of 20% FBS. For 24 h, the high serum concentration is necessary for the normal collapsing and proliferation of CMs.

1. Follow instructions for **Subheading 3.2.1.**, **steps 1–4**.
2. Change the medium to 1X SRM2/IMDM.
3. Continue with instructions from **Subheading 3.2.1.**, **steps 6–8**.

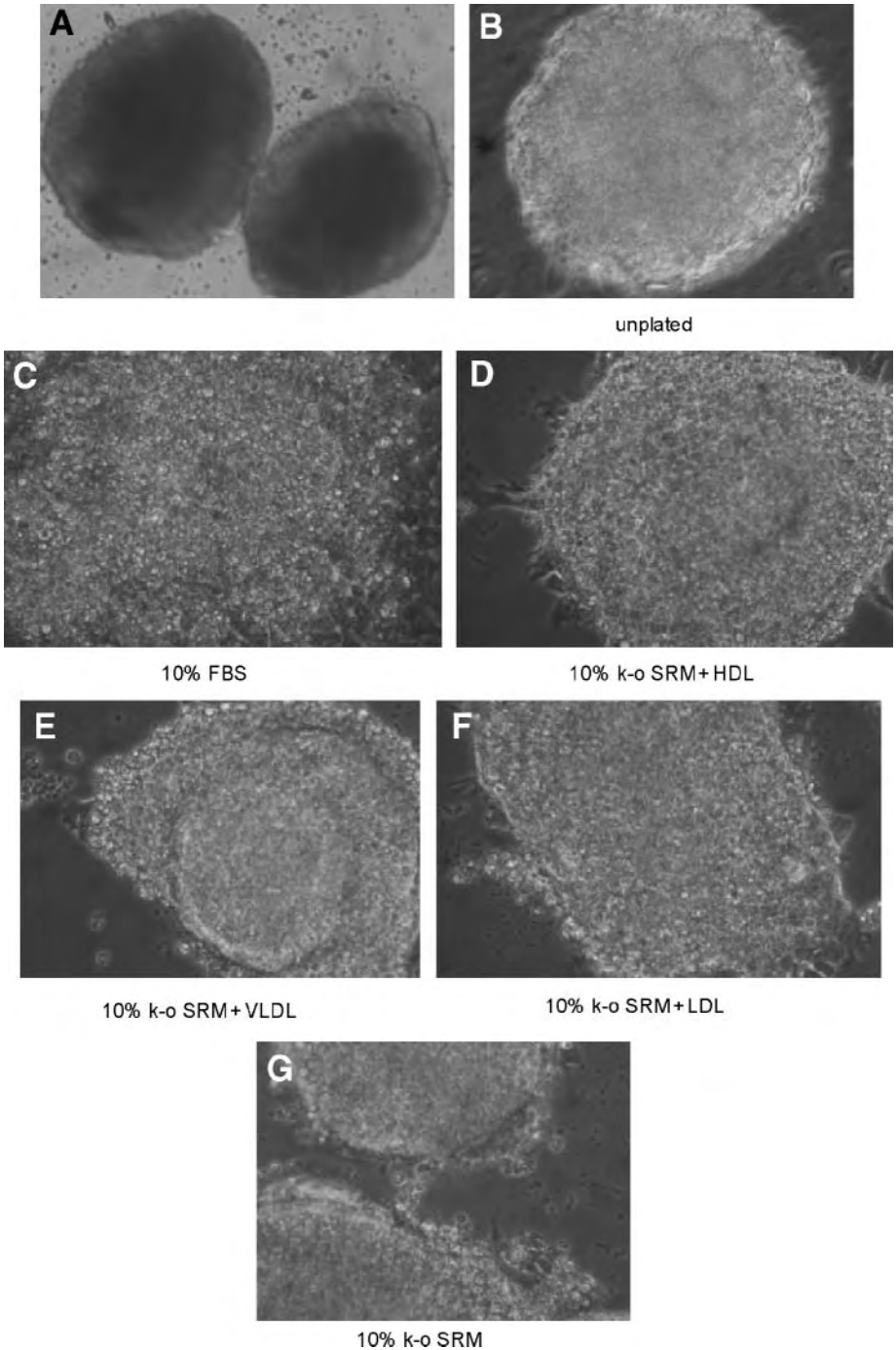
A
B
unplated
C
D
10% FBS
10% k-o SRM+HDL
E
F
10% k-o SRM+VLDL
10% k-o SRM+LDL
G
10% k-o SRM

Fig. 5.

3.2.3. Second Variation of the Standard Protocol

The second variation of the standard protocol requires serum-free cultured ES cells using the medium described under **Subheading 2.1.2.**, **item 2** (*see* **Note 27**). Serum-free generated and cultured EBs will normally not collapse or adhere to the plates. This effect will disappear when the plated EBs are cultured in serum-free starvation medium supplemented with lipoproteins (**Subheading 2.1.2.**, **item 8**) as shown in **Fig. 5**.

1. Prepare serum-free ES cell cultures as described in **Subheadings 3.1.5.** and **3.1.6.**
2. Prepare serum-free EBs as described in **Subheading 3.1.7.** using k-o EB culture medium.
3. Follow the instructions as described in **Subheading 3.2.1.**, **steps 2–4**, and plate the EBs in k-o EB culture medium or the serum-free starvation medium.
4. Continue with the instructions from **Subheading 3.2.1.**, **steps 6–8**.

3.2.4. The Third Variation of the Standard Protocol

The third variation of the standard protocol requires serum-free cultured ES cells and EBs (**Subheading 2.1.2.**, **items 2** and **5**). The EBs can be plated or cultured as swimming spheroids in bacteriological dishes. The cardiac differentiation is promoted by k-o DMEM supplemented with FBS.

1. Prepare serum-free ES cell cultures as described in **Subheadings 3.1.5.** and **3.1.6**.
2. Prepare serum-free EBs as described in **Subheading 3.1.7.** using k-o EB culture medium.
3. Follow the instructions as described in **Subheading 3.2.1.**, **steps 2–4**, plating the EBs in k-o starvation medium (*see* **Note 28**).
4. Continue with the instructions from **Subheading 3.2.1.**, **steps 6–8**.

3.3. Methods in Molecular Analysis

3.3.1. Sample Preparation

1. Prepare the samples as described in **Fig. 2A–E**.
2. Isolate total RNA using the Tri-Reagent kit following the manufacturer's instructions.
3. Synthesize cDNA using the ImProm kit II following manufacturer's instructions. Prepare 20-µL reactions with the Oligo-dT and random primer corresponding to 1 µg total RNA.

Fig. 5. Serum-free generated embryoid bodies (EBs) 24 h after plating in different starvation media. In general, serum-free generated EBs require a special stimulus for attachment after plating. Under serum-free conditions, cellular proliferation is reduced, and collapsing and growth of the EBs are minimized. We used different starvation media to overcome this problem: (**A**) 4-d-old EBs generated from serum-free embryonic stem cell cultures under serum-free conditions; (**B**) 5-d-old EBs in supplemented knockout Dulbecco's modified Eagle's medium (k-o DMEM) medium that in turn was supplemented with 10% k-o serum replacement media (k-o SRM); (**C**) 5-d-old EBs plated in supplemented k-o DMEM medium that in turn was supplemented with 10% fetal bovine serum; (**D**) 5-d-old EBs plated in supplemented k-o DMEM medium and supplemented with 10% k-o SRM and high-density lipoprotein (HDL); (**E**) 5-d-old EBs plated in supplemented k-o DMEM medium also supplemented with 10% k-o SRM and the very low-density lipoprotein; (**F**) 5-d-old EBs plated in supplemented k-o DMEM medium also supplemented with 10% k-o SRM and the low-density lipoprotein; (**G**) 5-d-old EBs plated in supplemented k-o DMEM medium also supplemented with 10% k-o SRM.

3.3.2. The PCR Analysis

1. Prepare PCR mix: 1 µL synthesized cDNA (corresponding to 50 pg total RNA), 6 µL 25 m*M* $MgCl_2$, 5 µL 10X PCR buffer, 50 pmol forward primer, 50 pmol reverse primer, 10 mmol dNTPs, 2.5 U *Taq* polymerase, and double-distilled water to the final volume of 50 µL.
2. Run the following PCR program: 10 min at 94°C, followed by 35 cycles for 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C and a final incubation for 10 min at 72°C.
3. Analyze 15 µL of each PCR product on a 2% agarose gel incorporated with ethidium bromide.
4. Examine the amplification products under the UV transilluminator as shown in **Fig. 3C–F**.

4. Notes

1. This does not require mitotically inactivated fibroblasts (called feeder layers) for its propagation in culture in the undifferentiated state.
2. L-Glutamine remains stable at 37°C for only a maximum of 5 d, and medium should be prepared fresh every time.
3 The diluted β-Mercaptoethanol remains stable for only a maximum of 5 d, and medium should be prepared fresh every time.
4. The nucleotide solution should be warmed for 30 min at 37°C before use.
5. Prepare only the necessary amounts of complete medium for just 5 d, corresponding to the lifetime of β-mercaptoethanol and L-glutamine.
6. The k-o SRM can also be reduced for the incubation of plated EBs to 10% k-o SRM, but for generation of EBs, 20% k-o SRM is required.
7. This is to reduce the amount of expensive substances like serum and LIF.
8. Common practice of cell culture is important to maintain sterile conditions and to avoid any contamination. According to the given rules in cell culture, always ensure the use of clean and sterile materials.
9. This is filled each week with autoclaved double-distilled water.
10. Take care while handling DEPC. It is a strong mutagen. Wear protective gloves and handle inside a chemical hood. DEPC can be destroyed by autoclaving.
11. Freezing and thawing of the cells is generally tricky. Therefore, always freeze cells stepwise slowly and thaw them rapidly.
12. We never checked if CGR8 ES cells can be cryopreserved under serum-free conditions in a cryoconservation medium in which the FBS is replaced by k-o SRM.
13. Wear protective clothes for this step.
14. Take care that thawing is rapid.
15. The cells should be homogenized by pipetting gently up and down. This is the trickiest step. Do it as quickly as possible and only as necessary.
16. Fill according to the safety instructions.
17. ES cells, which were passaged more than 12 times in a single stretch, often seem to start differentiation. To prevent this, freeze and thaw cells during propagation. For the generation of EBs, you should use only ES cells passaged more than three times after thawing.
18. Trypsin is a powerful enzyme that can destroy the cells. Therefore, never incubate the cells for more than 5 min in trypsin-EDTA solution.
19. Sometimes, ES cells will form clumps (aggregates). These clumps should be removed by passing the suspension through sterile 100-µm and 50-µm mesh; otherwise, the clumps will start to differentiate even in the presence of LIF.
20. Cell titer (cells/mL) is the number of counted cells times the chamber factor (Neubauer chamber factor = 10^4). Total number of cells = Cell titer × Volume of cell suspension used.

Volume to be used to resuspend the pellet = Total number of ES cells/1 × 10^6. To evaluate the viability of your cells, trypan blue can be added to the aliquot reserved for cell counting. White cells surrounded by a brown ring are live cells; dead cells are blue because of membrane rupture. If the suspension is not well homogenized, then the counting will not be exact, and the EBs will differ in size. The normal titer should be from 2 × 10^4 cells/mL to 2.5 × 10^4 cells/mL, corresponding to 400–500 cells/hanging drop (20 µL/drop).

21. Do not passage ES cells within 48 h after plating/replating.
22. Stopping the reaction by addition of serum is not possible in this case. The cellular adhesion is a Ca^{2+}-/Mg^{2+}-dependent reaction.
23. Do the entire procedure as quickly as possible and wash the cells before plating once with serum-free wash medium.
24. For generation of EBs, use only bacteriological Petri dishes because the inner surfaces of cell culture dishes are treated with special agents that make ES cells adhere to the surface.
25. It is important to be careful when plating the EBs. If more than 20 EBs/well of a 24-well plate are plated, then the cells will produce more CO_2, which increases the acidity of the medium. This decrease in pH value can reduce the cellular proliferation. Moreover, L-glutamine, MEM, and glucose are reduced by increased metabolism.
26. Counting the plated EBs is mandatory to obtain comparable experimental conditions in each well.
27. The EBs need serum or a starvation medium for the normal collapsing and proliferation after plating.
28. Note that the plating procedure is not required for obtaining beating CMs. Therefore, place the EBs in nongelatin-coated bacteriological dishes filled with k-o starvation medium (**Fig. 2E**).

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15

Analysis of Arrhythmic Potential of Embryonic Stem Cell-Derived Cardiomyocytes

Lijuan L. Shang, Samuel C. Dudley, Jr., and Arnold E. Pfahnl

Summary

By directed differentiation using the hanging drop method, cardiomyocytes (CMs) can be derived from mouse embryonic stem cells. These spontaneously active CMs can then be isolated from the embryoid bodies and studied electrophysiologically for analysis of arrhythmic potential. This method is particularly advantageous for studying CMs derived from genetically modified stem cells, in which mutations result in embryonic lethality.

Key Words: Action potential; arrhythmia; automaticity; cardiomyocyte; delayed afterdepolarization; early afterdepolarization; electrophysiology; embryonic stem cell; embryoid bodies; hanging drop; ion channel; patch-clamp; reentry; tissue culture; voltage gated.

1. Introduction

Cardiac stem cell therapy holds great promise to improve injured hearts *(1)*. Nevertheless, stem cell-derived cardiomyocytes (CMs) are excitable cells and have the potential to cause arrhythmias. The clinical data are mixed so far using adult cells *(2)*, and this arrhythmic potential may be more important in totipotent cells, which probably have greater potential to generate CMs *(3,4)*. Unfortunately, relatively few data exist on the electrophysiology of embryonic stem cell (ESC)-derived CMs or the consequences of their use in vivo. Comparing electrophysiological properties of ESC-derived and native-derived CMs could help determine if stem cells are likely to contribute to arrhythmic risk *(5)*.

Given the difficulty to isolate CMs differentiated in vivo, one approach to this problem is to study ESC-derived CMs differentiated in vitro. This approach is suitable also for studying genetic alterations in ESCs that result in embryonic lethality. In this chapter, we discuss the derivation of CMs from ESCs. Further, we discuss the isolation of

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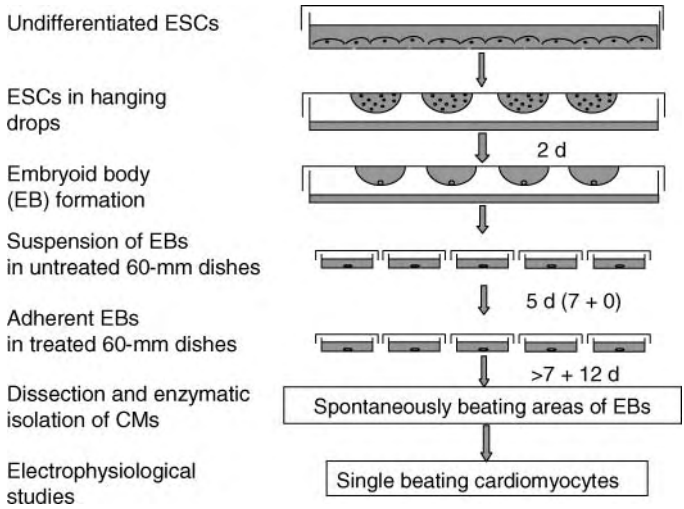

Fig. 1. A general scheme for embryonic stem cell (ESC) differentiation in vitro. For 2 d, undifferentiated ESCs are cultivated in hanging drops to form embryoid bodies (EBs). EBs are cultured in suspension for an additional 5 d in untreated 60-mm cell culture dishes. Spontaneously beating cardiomyocytes (CMs) can be observed under a microscope 2 d after plating the EBs onto treated 60-mm dishes (i.e., 7 + 0 d). Beating areas of EBs are mechanically dissected, and single CMs are enzymatically isolated after at least 7 + 2 d in the murine system to allow for complete differentiation. Single CMs are suitable for electrophysiological study.

single CMs suitable for electrophysiological study by the patch-clamp technique. A general scheme is shown in **Fig. 1**.

2. Materials

2.1. Tissue Culture

1. 100 mL 2-mercaptoethanol (1000X; Gibco Invitrogen, Carlsbad, CA; cat. no. 21985).
2. 50 mL cell culture freezing medium (1X) (dimethyl sulfoxide [DMSO] prepared in Dulbecco's modified Eagle's medium [DMEM] with fetal bovine serum [FBS] and calf serum) (Gibco Invitrogen, cat. no. 11101).
3. 1 g collagenase B (Roche, Indianapolis, IN; cat. no. 1088823).
4. 1 L high-glucose DMEM (1X with L-glutamine, 110 mg/L sodium pyruvate, and pyridoxine hydrochloride) (Gibco Invitrogen, cat. no. 11995).
5. 500-mL ESC-qualified FBS (performance, mycoplasma, virus, and endotoxin tested) (Gibco Invitrogen, cat. no. 10439).
6. 500 mL FBS (Gibco Invitrogen, cat. no. 10082).
7. 10 µg/mL leukemia inhibitory factor (LIF) (Chemicon, Temecula, CA; cat. no. LIF2010).
8. 200 m*M* L-glutamine (100X; Gibco Invitrogen, cat. no. 25030).
9. 100 mL minimum essential medium (MEM) amino acids solution (50X without L-glutamine) (Gibco Invitrogen, cat. no. 11130).
10. 4 mL mitomycin C (Sigma-Aldrich, St. Louis, MO; cat. no. M0503). To make the stock solution, dissolve 2 mg mitomycin C in 10 mL phosphate-buffered saline (PBS) and filter-sterilize with 0.2-µm filters. Store at 4°C or in aliquots at –20°C.

11. 100 mL penicillin-streptomycin (pen-strep) (100X; Gibco Invitrogen, cat. no. 15140-122).
12. PBS (1X): prepare 500 mL from 10X PBS, pH 7.4, without calcium chloride and magnesium chloride (Gibco Invitrogen, cat. no. 70011).
13. 2.5% trypsin (10X): prepare 100 mL with 25 g trypsin and 8.5 g NaCl per liter (Gibco Invitrogen, cat. no. 15090).
14. 100 nL trypsin-ethylenediaminetetraacetic acid (EDTA) (1X): 0.25% trypsin, 1 m*M* EDTA • 4 Na in Hanks balanced salt solution without Ca^{2+} and Mg^{2+} (Gibco Invitrogen, cat. no. 25200).

2.1.1. Media

1. Murine embryonic fibroblast (MEF) feeder cells are maintained in supplemented DMEM filtered using a 0.2-µm nitrocellulose filter. To prepare 500 mL MEF medium, add 50 mL FBS (heat inactivated), 5 mL L-glutamine (200 m*M*), 5 mL nonessential amino acids (10 m*M* each), 5 mL pen-strep (100X) into 435 mL DMEM. This medium is called 10% DMEM. Generally, this medium can be used for up to 2 wk.
2. R1 ESCs are maintained in 15% FBS DMEM. To make 500 mL, place 75 mL FBS (heat inactivated and ES tested), 5 mL L-glutamine (200 m*M*), 5 mL nonessential amino acids (10 m*M* each), 5 mL pen-strep (100X), and 0.5 mL 2-mercaptoethanol into 409.5 mL DMEM and filter by a 0.2-µm nitrocellulose filter. This medium can be used for 2 wk. A 10X stock solution of LIF must be added to the ESCs at all times: add 100 µL to 9.9 mL 15% DMEM.
3. ES freezing medium: DMEM supplemented with 20% FBS and 10% DMSO.
4. Medium for hanging drops: mouse R1 ESCs are cultured in 20% FBS DMEM for forming embryonic bodies (EBs) using the hanging drop technique ***(6)***. To make 500 mL, place 100 mL FBS into 384.5 mL DMEM. Also, add 5 mL L-glutamine (200 m*M*), 5 mL nonessential amino acids (10 m*M* each), 5 mL pen-strep (100X), and 0.5 mL 2-mercaptoethanol.

2.1.2. Single Beating CM Isolation

1. 15% DMEM supplemented buffer for ESC culture (*see* **Subheading 2.1.1.**, **item 2**).
2. 35-mm cell culture dishes (Corning, Acton, MA; cat. no. 430165).
3. 1 g collagenase B (Roche; cat. no. 1088823). Ca^{2+}-free digestion solution of collagenase B for single beating cell isolation: for 100 mL, mix 0.7 mg NaCl, 40 mg KCl, 123 mg $MgSO_4$, 55 mg Na pyruvate, 360 mg glucose, 250 mg taurine, and 238.3 mg 10 m*M* HEPES. Add NaOH and adjust to pH 6.9. Use with collagenase B (filtered) at 1 mg/mL for a final concentration.
4. 25-mm cover slips (Sarstedt, Newton, NC; cat. no. 83.1840).
5. Tungsten dissection probe (50 mm long, 0.5-mm diameter rod; World Precision Instruments [WPI], Sarasota, FL; cat. no. 5001350).
6. Handles for probe (11 cm long, stainless steel; WPI, cat. no. 500448).
7. Krebs buffer (KB): for 100 mL, mix 633 mg KCl, 522 mg K_2HPO_4, 123 mg $MgSO_4$, 38 mg EDTA, 121 mg adenosine triphosphate (ATP), 55 mg pyruvic acid, 74.6 mg creatine, 250.4 mg taurine, and 360.4 mg glucose. Make stock without ATP and store at –20°C in small (0.5- to 1.0-mL) aliquots. Add fresh ATP when needed.
8. PBS (1X; *see* **Subheading 2.1.**, **item 12**).
9. 1.7-mL microcentrifuge tube (Corning, cat. no. 3621).

2.2. Patch-Clamp Recordings

2.2.1. Media and Solutions

1. Bath solution (external solutions): the culture medium is replaced by the recording bath solution shortly before the experiment. The bath solution consists of 140 m*M* NaCl (8.1816 g/L),

5.4 m*M* KCl (0.9091 g/L), 1.8 m*M* $CaCl_2$ (0.2646 g/L), 1 m*M* $MgCl_2$ (0.0952 g/L), 10 m*M* HEPES (2.3830 g/L), and 10 m*M* glucose (1.8016 g/L) (pH 7.4 by 1 *M* NaOH) at 37°C.

2. Patch-Clamp Glass Pipet Filling Solutions (Internal Solutions):
   a. For ruptured patches to record action potentials, a reasonable filling solution consists of 120 m*M* KCl (0.8946 g/100 mL), 1 m*M* $MgCl_2$ (0.0095 g/100 mL), 3 m*M* MgATP (0.1522 g/100 mL), 10 m*M* HEPES (0.2383 g/100 mL), and 10 m*M* EGTA (0.3804 g/100 mL) (pH 7.2 by 1 *M* KOH).
   b. For perforated patches, the pipet solution contains 120 m*M* KCl (0.8946 g/100 mL), 1 m*M* $MgCl_2$ (0.0095 g/100 mL), 3 m*M* MgATP (0.1522 g/100 mL), 10 m*M* HEPES (0.2383 g/100 mL), and 10 m*M* EGTA (0.3804 g/100 mL) plus an ionophore (e.g., amphotericin B, gramicidin D, or nystatin) or a saponin (e.g., β-escin) (pH 7.2 by 1 *M* KOH).

2.2.2. Patch-Clamp Accessories

1. Glass pipets for patch-clamp recordings: patch-clamp capillary glass (WPI, cat. no. PG52150-4) is pulled to a resistance of 2–5 MΩ on a P-97 Flaming/Brown Micropipette Puller (Novato, CA). The pipet tips are polished on a Narishige polisher (Narishige, Tokyo, Japan; model no. MF-830).
2. Patch-clamp apparatus: action potentials can be recorded in the whole-cell, single-electrode, current-clamp configuration using an Axopatch 200B amplifier and a Digidata 1320 analog-to-digital interface (Axon Instruments, Burlingame, CA). *pCLAMP* software (Axon Instruments) can be used to generate stimulation protocols and acquire, store, and analyze data.
3. Patch-clamp perfusion system: the perfusion system is critical to obtaining good recordings from CMs because CM action potentials are sensitive to temperature. The perfusion system maintains tissue chamber temperature at a constant 37°C and allows the superfusion of various test solutions. A suitable system, including a temperature-controlled tissue chamber, perfusion controller, and perfusate prewarmer, can be obtained from Cell Micro Controls (Norfolk, VA).

2.2.3. Commonly Used Test Chemicals for Patch-Clamping

1. Nifedipine (Sigma, cat. no. N-734) is used to block L-type calcium channels. DMSO can be used to dissolve nifedipine up to a concentration of 50 mg/mL. Usually, a stock solution of 10 m*M* in DMSO is made and used at a working concentration of 1–3 μ*M*. To make the 10 m*M* stock, weigh out 34.63 mg nifedipine powder and dissolve in 1 mL DMSO. To make 1 μ*M* nifedipine working concentration, add 1 μL stock solution to 10 mL patch-clamp bath solution.
2. Bay K8644 (Sigma, cat. no. B-133) is used to activate L-type calcium channels. Make a 2 m*M* stock in DMSO. Working concentration is 1 μ*M*. To make the 2 m*M* stock, weigh out 0.71 mg Bay K8644 powder and dissolve in 1 mL DMSO. To make 1 μ*M* Bay K8644 working concentration, add 5 μL stock solution to 10 mL patch-clamp bath solution.
3. Ryanodine (Sigma, cat. no. R 6017) is used to block Ca^{2+} release from the sacroplasmic reticulum at 10 μ*M*. Lower doses may increase Ca^{2+} release, however. To make a 60 m*M* stock solution in DMSO, weigh out 29.67 mg ryanodine powder and dissolve in 1 mL DMSO. To make 10 μ*M* ryanodine working concentration, add 1.7 μL stock solution to 10 mL patch-clamp bath solution.
4. Tetrodotoxin (TTX) (Sigma, cat. no. T-5651) blocks sodium channels: the TTX powder is dissolved in 50 m*M* acetic acid with a working concentration of 3 μ*M*. To make the 50 m*M* stock, weigh out 15.96 mg TTX powder and dissolve in 1 mL glacial acetic acid. To make 3 μ*M* TTX working concentration, add 0.6 μL stock solution to 10 mL patch-clamp bath solution.

5. Tetraethylammonium chloride (TEA) (Sigma, cat. no. T-2265) blocks K^+ channels: the stock concentration is 2 *M*; dilute to a working concentration of 20 m*M*. To make the 2 *M* stock, weigh out 331 mg TEA powder and dissolve in 1 mL H_2O. To make 20 m*M* TEA working concentration, add 100 µL stock solution to 10 mL patch-clamp bath solution.

3. Methods

3.1. Tissue Culture (see Note 1)

3.1.1. Freezing Cells

Because of the length of time involved, in vitro differentiation is subject to contamination and other errors. Therefore, it is wise to have backup frozen ESCs.

1. When ESCs are ready to freeze, transfer the cells into a 15-mL tube and pellet the cells by centrifuging at 200*g* for 3 min.
2. Remove the supernatant completely and add freezing medium to obtain a final concentration at $3–5 \times 10^6$ cells/mL.
3. Triturate to dissociate the cells and transfer 1 mL cell suspension into each cryovial.
4. Put cryovials into Nalgene cryoware box and place the box into an –80°C freezer, allowing the cells to freeze slowly.
5. The next day, transfer the vials of frozen cells into a liquid nitrogen storage tank.

3.1.2. Thawing Cells

1. Thaw frozen vials of cells in a 37°C water bath.
2. When the cells are completely thawed, clean the vial with 70% ethanol and transfer the contents into a flask containing culture medium.
3. Place the cells in a 37°C, 5% CO_2, 86% humidity incubator (*see* **Note 2**).

3.1.3. Mitomycin C Treatment of Feeder Layer for ESCs

The first step in culturing totipotent stem cells is to have a healthy feeder cell layer established in a 70-mL vented flask. Feeder cells provide LIF and otherwise maintain the undifferentiated state. Feeder cells can be obtained commercially or from pregnant mice using a standard protocol. The feeder cell division is arrested with mitomycin C prior to ESC plating so that the feeder cells do not overgrow the more slowly dividing ESCs. The primary feeder culture can be passaged a maximum of five times.

1. Remove medium from the flask with confluent feeder cells and replace it with 6 mL fresh medium.
2. Add 300 µL mitomycin C (*see* **Note 3**).
3. Incubate at 37°C for 2–3 h.
4. Remove the medium with mitomycin C and wash the cells with PBS three times, then add fresh 15% DMEM. Now, the feeder cells are ready to support ESCs.

3.1.4. Culturing ESCs

1. Frozen ESCs are stored in freezing medium. Frozen cells (1 mL) are added to 10 mL 15% DMEM and centrifuged at 200*g* for 3 min. Remove the supernatant and add fresh 15% DMEM with LIF (*see* **Note 4**). The thawed ESCs are seeded onto a mitomycin C-treated flask of feeder cells. Start the ESC culture as in the following steps.

2. Culture ESCs at 37°C for 48 h to form the ESC clones.
3. Check the cells the next day to determine if fresh medium is required (necessary if the medium color turns yellow or there are excessive numbers of nonadherent cells).
4. When the ESC colonies are large, passage 1 to 2. This may take 2–3 d.
5. To passage, remove ES medium, wash plates with PBS once, and add 1.5 mL 0.25% trypsin/EDTA. Incubate at 37°C for 2–3 min. Add 10 mL ES medium and pipet vigorously to break up all the clumps.
6. Add 5 mL to each of two feeder flasks containing 5 mL ES medium. At this point, if you have excess ESCs, then you may freeze them at 2–5 × 10^6 cells per vial.

3.1.5. In Vitro Differentiation of ESCs Into Cardiomyocytes

3.1.5.1. Preparation of Hanging Drops of ESCs

1. To each 10-cm Petri dish, add 6–8 mL sterile PBS and label the lid of the dish accordingly.
2. Treat the plates with trypsin/EDTA at 37°C for 2–3 min. Stop the reaction by adding culture medium. Spin down the cells.
3. Remove the supernatant and resuspend the cells with 10 mL 20% DMEM.
4. Perform a cell count. The small cells are the ESCs. Cells must be diluted to a concentration of 900 cells/30 µL medium.
5. Add 30 µL diluted cell suspension to the lid of the Petri dish to create a single hanging drop. Multiple drops can be placed on each lid. Lids are replaced on the top of Petri dishes containing PBS for incubation.

3.1.5.2. Suspending and Plating of EBs

Two days after creation and incubation, the hanging drops must be washed down into a suspension culture.

1. Wash down hanging drops with 5 mL fresh DMEM.
2. With EBs in suspension, incubate undisturbed for another 5 d.
3. Then, plate EBs on treated 60-mm cell culture dishes, 5–10 EBs in each dish.

3.2. Troponin T Immunohistochemical Staining for Cardiomyocytes

Although CMs are recognized usually by their striations and spontaneous contractile activity, immunohistochemistry can be used to identify fixed CMs. Troponin T is a thin filament regulatory protein of striated muscle that binds to troponin C and I as well tropomyosin, which is required for calcium-dependent adenosine triphosphatase activity of the myofibrillar proteins. The antibody ab-1 specifically recognizes the cardiac isoform of troponin T, allowing for identification of CMs (**Fig. 2**).

3.3. Isolation of Single Beating Cardiomyocytes

1. Dissect out beating areas from EBs using the tungsten dissection probe under a stereomicroscope and place isolated parts of the EBs in PBS at room temperature for no more than 30 min.
2. Once 5–10 beating areas are successfully dissected, spin down the tissue at 200*g* for 3 min to remove PBS.
3. Add 70 µL collagenase mix containing 30 µ*M* $CaCl_2$. Incubate at 37°C for 30 min.
4. Carefully remove the collagenase solution, leaving behind the tissue. To the vial containing the tissue, add 100 µL KB. Triturate EBs to dissociate the cells. Gently shake at room temperature for 1 h.

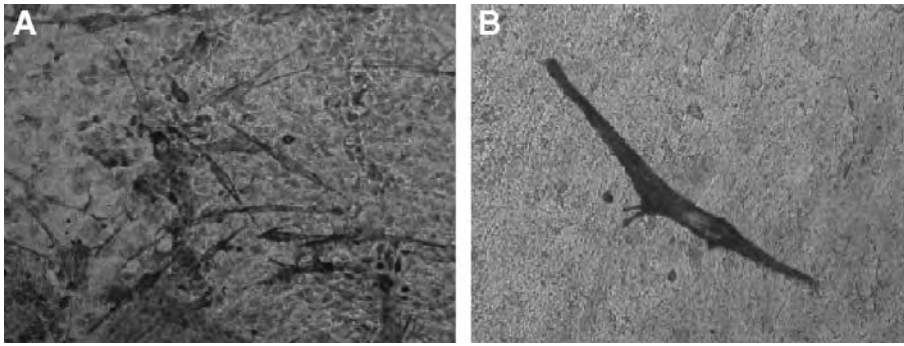

Fig. 2. Immunohistochemical characterization of cardiomyocytes (CMs) in differentiated embryonic bodies (EBs). **(A)** The distribution of CMs (dark) in the EBs (×20). **(B)** A single CM stained for troponin T, a cardiac-specific contractile protein (×40).

5. Take 100 µL of cells in the KB solution and add to 2–5 mL 15% DMEM medium. Take approx 300–600 µL cell suspension and place carefully onto a cover slip placed within a culture dish. Avoid allowing the drop to spread out into the dish to maximize the yield of cells on the cover slip.
6. Incubate at 37°C overnight.
7. The following morning, change the medium to remove vestiges of KB. Cells will not contract unless the medium is changed to fresh 15% FBS culture medium.

3.4. Electrophysiological Analysis of Arrhythmic Potential of ESC-Derived CMs

3.4.1. Identification of Beating CMs on Cover Slips

Isolated ESC-derived CMs are plated onto sterile cover slips. Plastic cover slips can be cut to size with scissors to fit the patch-clamp perfusion chamber. Glass cover slips can be broken to size with forceps or scored and broken to size.

3.4.2. Whole-Cell Patch-Clamp Technique

Action potentials are recorded from whole CMs using either the ruptured patch or perforated patch method.

3.4.2.1. Ruptured Patch Technique

In the ruptured patch technique, a high-resistance seal is made between the membrane and the electrode. The membrane underneath the electrode is ruptured with oscillating voltage or changes in hydrostatic pressure. This allows communication between the cytoplasm and the pipet filling solution. To allow equilibrium to be reached between the cytoplasm and pipet solution, it is important to wait 5–10 min after patch rupture. Calcium buffers are required to maintain an appropriately low calcium concentration in the cell; otherwise, the CMs contract and die quickly.

3.4.2.2. Perforated Patch Technique

In the ruptured patch technique, dialysis of the cytoplasm may alter electrical behavior. To avoid this, the perforated patch technique may be used. The perforated patch

method chemically creates numerous small holes in the patched cell membrane separating the electrode filling solution from the cytoplasm. This allows electrical connection without cytoplasmic dialysis. The chemicals used include amphotericin B, gramicidin D, β-escin, or nystatin.

3.4.2.3. Recording Action Potentials and Depolarizations From CMs

The extrapolation from cellular electrophysiology to arrhythmic risk is challenging. Classically, there are three arrhythmic mechanisms: (1) automaticity, by which spontaneous activity is enhanced; (2) triggered activity, by which one action potential initiates oscillatory electrical activity, leading to more action potentials; and (3) reentry, by which continuous electrical activity is established in the tissue. Reentry is favored by areas of slow conduction, and at the level of single cells, slow conduction is favored by reduced Na^+ channel availability, which is reflected in the maximum rate of rise of the action potential. These three mechanisms can be evaluated by analyzing both induced and spontaneous CM action potentials. To record these signals, the patch-clamp amplifier is set to the whole-cell, current-clamp mode. Induced action potentials and depolarizations are obtained by applying a stimulation protocol (i.e., pacing protocol) via Axon's *pClamp* software. Action potentials can be elicited by pacing with a 200-pA amplitude and 10-ms duration pulse. The frequency of pacing can be varied to determine the frequency dependence of changes in action potential properties.

3.4.2.4. Identification and Characterization of Cardiomyocyte Action Potentials

CM action potentials can be categorized into nodal, atrial, and ventricular-like action potentials based on the following properties (*see* **Note 5** and **Fig. 3**):

1. Maximum diastolic potential: more negative for atrial and ventricular CMs.
2. Maximum rate of action potential rise (dV/dt): faster for atrial and ventricular CMs. The fast rate of rise is mediated by voltage-gated sodium channels.
3. Action potential duration: longer for nodal and ventricular CMs.
4. Action potential overshoot: larger for atrial and ventricular CMs because of voltage-gated sodium channels. The slow rate of rise and reduced overshoot represent a lack of Na^+ channel current in the nodal type cells.
5. Plateau phase: longest in ventricular-type CMs.

In general, ESC-derived CMs are spontaneously active and show slowed maximum upstroke velocities and longer action potential durations than native cells. Also, specific beating cell morphologies are not associated with action potential properties.

3.4.2.5. Testing for Triggered Activity

Triggered arrhythmias arise from membrane oscillatory depolarizations that occur before or after full repolarization of the preceding action potential. These oscillations are called early afterdepolarizations (EADs) and delayed afterdepolarizations (DADs), respectively. Either spontaneous or induced triggered activity can be evaluated. Comparing rates during provocation can be more fruitful because the

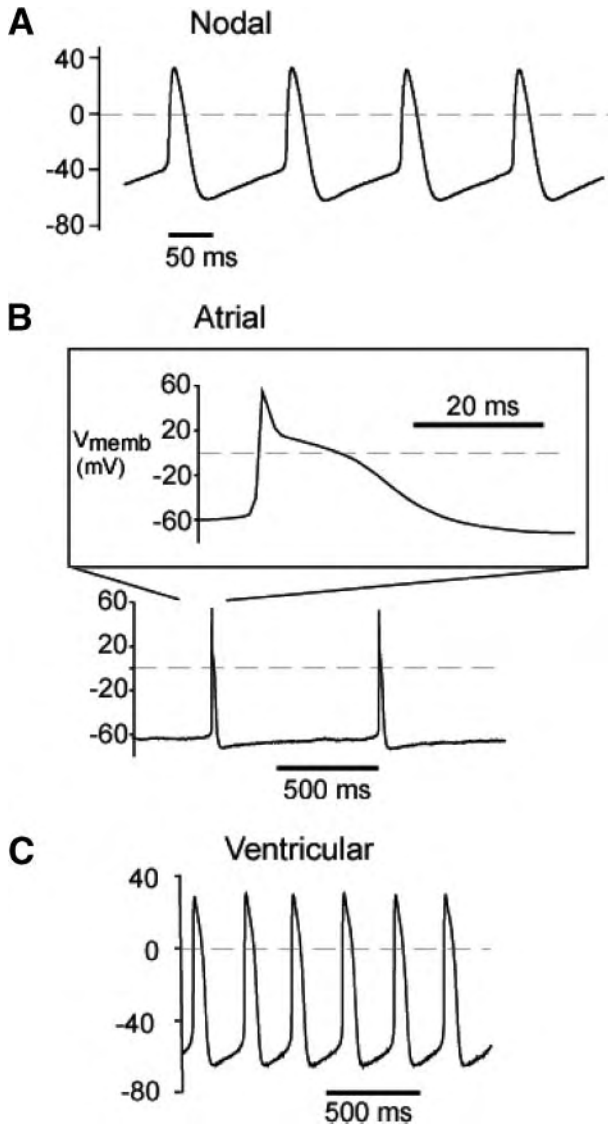

Fig. 3. Representative action potentials obtained from embryonic stem cell-derived cardiomyocytes. Notice the rate of AP upstroke (dV/dt) is slower for nodal (~5 mV/ms) than for other cardiomyocytes (~65 mV/ms). Action potentials were evoked by pulse current stimulation (200 pA, 10 ms) and recorded at 37°C in the whole-cell current clamp mode.

spontaneous rate of triggered activity is relatively low. EADs can be induced by slow pacing rates or action potential-prolonging drugs. Alternatively, DADs can be provoked by maneuvers that load the cell with Ca^{2+}. Examples of EAD and DAD are shown in **Fig. 4**.

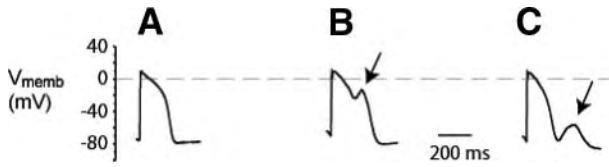

Fig. 4. Early afterdepolarizations (EADs) and delayed afterdepolarizations (DADs) recorded from embryonic stem cell (ESC)-derived cardiomyocytes (CMs). (**A**) An action potential from a CM perfused in regular bath medium showing reduced upstroke velocity and prolonged action potential duration when compared to native CMs. (**B**) An EAD (arrow) elicited from a CM treated with 1 μ*M* Bay K8644 and 20 m*M* Tetraethylammonium chloride to prolong the action potential further. (**C**) A DAD (arrow) elicited by exposure to 1 μ*M* isoproterenol. Triggered activity is more frequent in ESC-derived than in native CMs under similar conditions. Action potentials were elicited with a 200-pA, 10-ms pulse.

4. Notes

1. ESCs are cultured in a 37°C, 5% CO_2, 86% humidity incubator. All manipulations are performed in a laminar flow cabinet. Before work, turn the cabinet on for at least 15 min and clean with 70% ethanol or diluted bleach. Before using, culture medium should be warmed to 37°C in a water bath for 15 min. Aliquot stock FBS into 50-mL tubes. Aliquot trypsin into 15-mL tubes to avoid multiple freeze-thaw cycles.
2. Every frozen cell preparation thaws a little differently. For example, if feeder cells are only 50% confluent, then thaw another vial into your ongoing flask. It is important for the feeder cells to be maintained at a relatively high density.
3. Mitomycin C is a carcinogenic agent and should be treated with great care. While working in a fume hood, use gloves and a face shield to limit any contact.
4. If the cells are to be unattended for weekends, then it may be desirable to add extra LIF (1.5X stock solution) to suppress differentiation.
5. When using the ruptured patch method to record action potentials, assess the validity of the method by comparing to the perforated patch method.

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Derivation and Characterization of Alveolar Epithelial Cells From Murine Embryonic Stem Cells In Vitro

Ali Samadikuchaksaraei and Anne E. Bishop

Summary

We present a protocol that has been developed for induction of the differentiation of murine embryonic stem (ES) cells to alveolar type II cells. With this protocol, undifferentiated murine ES cells are induced to form embryoid bodies (EBs). The 10-d-old EBs are transferred to adherent culture conditions and are fed with high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v fetal bovine serum, 2 m*M* L-glutamine, and 0.1 m*M* 2-mercaptoethanol for 20 d without splitting. Then, the cells are fed with a medium designed for the maintenance and growth of mature distal airway epithelial cells (small airway growth medium, SAGM) for 3 d. Characterization of the alveolar type II cells was done using real-time reverse transcriptase polymerase chain reaction detection of surfactant protein C mRNA and immunocytochemical detection of prosurfactant protein C. Real-time reverse transcriptase polymerase chain reaction revealed that SAGM increases the mRNA expression level of SPC by a factor of 8 when compared to that of cells grown in supplemented high-glucose DMEM ($p < 0.05$, Student *t*-test). Immunocytochemistry revealed that proSPC-expressing cells comprised 2.8 ± 0.23% of the total cell population in SAGM-treated samples and 0.5 ± 0.1% in samples treated with supplemented high-glucose DMEM ($p < 0.05$, χ^2 test).

Key Words: Alveolar epithelial cells; differentiation; embryonic stem cells.

1. Introduction

Lung transplantation is an acceptable means of treating several end-stage lung diseases, but donor shortage is a major problem *(1–4)*. Over the past few years, the number of lung transplantations performed annually has reached a plateau *(5)* as the donor pool is approaching its limits and cannot be expanded. Thus, other sources of a replacement gas exchange unit are sought. Xenotransplantation *(6)* and bioartificial devices *(7–12)* are possible candidates. Another possibility would be to engineer a gas exchange unit with some viable components in vitro. This unit may have the potential to take the responsibilities of a real lung and therefore would decrease the need for donor lungs.

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Alveoli are the basic functional units of lungs and gas exchange occurs across their membranes. Therefore, the first step in tissue engineering of lung should be aimed at generation of alveolar tissue. The epithelium of an alveolus is an endoderm-derived tissue and is composed of two main cell types; type I and type II pneumocytes. It is known that if type I cells are lost following a peripheral lung injury, then type II cells undergo proliferation and differentiation to the type I phenotype *(13)*. Thus, type II cells are crucial to the natural regenerative process of the alveoli. In fact, type II cells are considered putative alveolar stem cells *(13,14)*. Therefore, generation of type II pneumocytes would be a good basis for tissue engineering of the alveolar functional unit.

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of the blastocyst *(15)* with extensive proliferative capabilities *(16)*. As ES cells have the potential to produce any desired adult cell in an unlimited number, they are proving to be an invaluable resource for tissue engineering purposes. Manipulation of the culture medium and constituent growth factors is one of the methods that have been widely used for differentiation of several cell types from ES cells *(17–33)*. For example, it has been shown that by providing a medium for mature bone cells it is possible to enhance the differentiation of osteoblasts from ES cells *(19)*.

Most protocols for directing ES cell differentiation involve formation of three-dimensional embryoid bodies (EBs) from ES cells at early stages of their differentiation. This is achieved by removal of all differentiation-inhibiting factors from the culture and keeping ES cells in suspension for several days. Using developmental marker genes, it has been shown that the temporal and spatial patterns of differentiation during EB formation recapitulates many processes that take place during normal mammalian peri- and early postimplantation development *(34)*. This is the rationale for using EBs in many experimental procedures as it leads to more reproducible and ordered generation of derivatives of all three germ layers *(35)*. By using the above basic principles, we describe protocols here for culture conditions in which EBs are directed to differentiate into alveolar type II cells by providing a medium designed for the maintenance and growth of mature distal airway epithelial cells.

2. Materials

2.1. Tissue Culture

1. Undifferentiated E14 Tg2a murine ES cell line (kind gift of Prof. A. Smith, Institute of Stem Cell Research, University of Edinburgh, UK).
2. STO/SNL murine embryonic fibroblast feeder cell line (kind gift of Dr. V. Episkopou, Mammalian Neurogenesis Group, MRC Clinical Sciences Centre, Imperial College, London, UK).
3. Culture flasks with tissue culture-treated low-toxin (endotoxin level < 0.5 endotoxin units per milliliter [EU/mL]) high-grade polystyrene culture surfaces (Orange Scientific, Braine-l' Alleud, Belgium; T-75 flasks, cat. no. 2020200; T-25 flasks, cat. no. 2010100).
4. Cell attachment resistance Petri dishes (Nalgene polymethylpentene Petri dishes [Nalge Europe Ltd., Hereford, UK], available through VWR International Ltd., Leics, UK; cat. no. 402/0073/00).
5. Leukemia inhibitory factor (LIF; ESGRO) (Chemicon, Temecula, CA; cat. no. ESG1106).
6. Gelatin solution type B from bovine skin 2% (Sigma-Aldrich, Dorset, UK; cat. no. G1393). Dilute the concentrated solution with tissue culture-grade phosphate-buffered saline (PBS)

to 0.1%. For example, increase the volume of 5 mL concentrated solution to 100 mL by tissue culture-grade PBS.
7. Dimethyl sulfoxide Hybri-Max (DMSO) (Sigma-Aldrich, cat. no. D 2650).
8. 1X tissue culture-grade PBS without calcium or magnesium (Gibco Invitrogen, Life Technologies, Paisley, UK; cat. no. 14190-185).
9. High-glucose Dulbecco's modified Eagle's medium (DMEM) (1X) contains 4500 mg/L glucose and pyridoxine hydrochloride, but no L-glutamine or sodium pyruvate (Gibco Invitrogen, Life Technologies, cat. no. 41965-039).
10. Heat-inactivated FBS characterized and screened for ES cell growth (batch tested) (Gibco Invitrogen, Life Technologies, cat. no. 10108-165).
11. 200 m*M* L-glutamine (Gibco Invitrogen, Life Technologies, cat. no. 25030-024).
12. Small airways growth medium (SAGM) (Cambrex BioScience Wokingham Ltd., Berkshire, UK; cat. no. CC-3118).
13. Mitomycin C (Sigma-Aldrich, cat. no. M 0503): prepare stock solution of mitomycin C by dissolving a 2-mg vial in 2 mL PBS. Add this to 18 mL culture medium to make a 10X mitomycin C solution. Wrap the container with foil to protect it from light. Use within 2 wk. Dilute 10X mitomycin C solution with supplemented high-glucose DMEM (*see* **Subheading 2.1.1., item 1**) to make 1X solution and warm it.
14. Trypsin-ethylenediaminetetraacetic acid (EDTA) solution: 0.05% trypsin/0.53 m*M* EDTA in 0.1 *M* PBS without calcium or magnesium (Gibco Invitrogen, Life Technologies, cat. no. 25300-054).
15. 15- and 50-mL polypropylene conical centrifuge tubes (VWR International, cat. no. 734/0451 and cat. no. 525/0124, respectively).
16. Cryogenic vials (VWR International, cat. no. 734-1854).
17. Tissue culture-tested distilled water (Gibco Invitrogen, Life Technologies, cat. no. 15230-089).
18. 14.3 *M* mercaptoethanol 2-(ME) (Sigma-Aldrich, cat. no. M-7522): dilute the solution into 100 m*M* by adding 70 µL concentrated solution into 9930 µL tissue culture-tested distilled water.
19. Ethanol 99.7–100% (absolute) GPR (VWR International Ltd, cat. no. M-7522).
20. Virkon (VWR International, cat. no. 222/0154/02).
21. Geneticin (G418) (Gibco Invitrogen, Life Technologies, cat. no. 10131-027).
22. 100% isopropyl alcohol bath (Nalgene Mr. Frosty; Nalge Europe Ltd., Hereford, UK; cat. no. 5100).

2.1.1. Media

1. Supplemented high-glucose DMEM: supplement the high-glucose DMEM with 10% v/v fetal bovine serum (FBS), 2 m*M* L-glutamine, and 0.1 m*M* 2-ME immediately before use. For example, add 50 mL FBS and 5 mL L-glutamine to 450 mL high-glucose DMEM. Add 1 µL 100 m*M* 2-ME per milliliter of the culture medium immediately before use (*see* **Note 1**).
2. Freezing medium: prepare freezing medium, which consists of FBS and 10% v/v DMSO.
3. Inactivation medium: this medium consists of supplemented high-glucose DMEM (*see* **Subheading 2.1.1., item 1**) plus 200 µg/mL geneticin (G418) sulfate.

2.1.2. General Cell Maintenance Procedures

The following maintenance is applicable to many cell types and cell culture media.

1. Maintain the cells in an incubator at 37°C in a humidified atmosphere of 5% CO_2/95% air.
2. Control the water level in the incubator's tank daily.

3. Check cells daily under inverted microscope. If any contamination is observed in the cultured cells, then take the culture plate out of incubator and apply 10% Virkon for approx 1–2 h and then discard the plate.
4. After using a tissue culture hood, wipe it with 1% Virkon and aspirate some 10% Virkon for decontamination of tube and contents of canister.
5. Discard contents of canister after 1–2 h treatment with Virkon.

2.2. Reverse Transcriptase Polymerase Chain Reaction

1. Reverse transcriptase polymerase chain reaction (RT-PCR)-grade water (Ambion [Europe] Ltd., Cambridgeshire, UK; cat. no. 9935).
2. Total RNA Isolation System (Promega UK Ltd., Southampton, UK; cat. no. Z 3100). This kit contains the following ready-made buffers:
   a. Lysis buffer: 4 *M* guanidine thiocyanate, 0.01 *M* Tris-HCl (pH 7.0), and 97% β-mercaptoethanol (v/v).
   b. DNase stop solution: 2 *M* guanidine thiocyanate, 7 m*M* Tris-HCl (pH 7.5), and 57% ethanol (v/v).
   c. Wash solution: 60 m*M* potassium acetate, 10 m*M* Tris-HCl (pH 7.5), and 60% ethanol (v/v).
   d. Yellow core buffer: 0.0225 *M* Tris-HCl (pH 7.5), 1.125 *M* NaCl, and 0.0025% yellow dye (w/v) (the dye has no effect on the quality or the downstream performance of the RNA).
3. Trypsin/EDTA solution (0.05% trypsin/0.53 m*M* EDTA in 0.1 *M* PBS without calcium or magnesium) (Gibco Invitrogen, Life Technologies, cat. no. 25300-054).
4. Tissue culture-grade 1X PBS without calcium or magnesium (Gibco Invitrogen, Life Technologies, cat. no. 14190-185).
5. RNase-, DNA-, adenosine triphosphate (ATP)-, and pyrogen-free 1.5-mL Eppendorf tubes (VWR International, cat. no. 306/0329/02).
6. Spectrophotometer (Eppendorf BioPhotometer, Eppendorf-Netheler-Hinz, Hamburg, Germany).
7. TE buffer at pH 7.0 (Ambion, cat. no. 9861).
8. Molecular biology-grade ethanol (VWR International, cat. no. 43743 3T).
9. MessageSensor RT kit (Ambion, cat. no. 1745). This kit contains the following reagents:
   a. Ready-made 10X RT-PCR buffer containing 100 m*M* Tris-HCl at pH 8.3, 500 m*M* KCl, and 15 m*M* $MgCl_2$.
   b. 2.5 m*M* dNTP mix.
   c. RNase inhibitor.
   d. M-MLV-RT.
10. Random decamers (random primer) (Ambion, cat. no. 5722G).
11. Glycogen (Ambion, cat. no. 9510).
12. Poly(C) (Amersham Biosciences, Buckinghamshire, UK; cat. no. 27-4220-02).
13. Poly(dC) (Amersham Biosciences, cat. no. 27-7838-02).
14. 2 *M* sodium acetate buffer (pH 4.4–4.6) (AB Applied Biosystems, Warrington, UK; cat. no. 400884).
15. SYBR green PCR core reagents (AB Applied Biosystems, cat. no. 4304886). This kit contains:
   a. Ready-made 10X SYBR green PCR buffer.
   b. AmpErase UNG containing 1 U/µL uracil-*N*-glycosylate.
   c. 5 U/µL AmpliTaq Gold DNA polymerase.
   d. dNTP mix containing 2.5 m*M* dATP, 2.5 m*M* dCTP, 2.5 m*M* dGTP, and 5.0 m*M* dUTP.
   e. 25 m*M* $MgCl_2$.

16. GeneAmp 5700 Sequence Detection System (AB Applied Biosystems).
17. Primers for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (108-bp amplicon size):
    a. Forward primer: AGAAGGTGGTGAAGCAGGC.
    b. Reverse primer: AGGTGGAAGAGTGGGAGTTG.
18. Primers for the surfactant protein C (SPC) gene (specific marker for alveolar type II cells; *38–45*) (145-bp amplicon size):
    a. Forward (exon 2): TTGTCGTGGTGATTGTAGGG.
    b. Reverse (exon 3): AAGGTAGCGATGGTGTCTGC.
19. Mouse lung total RNA (Ambion, cat. no. 7818).

2.3. Immunocytochemistry

1. Magnetic stirrer (Corning Inc., Acton, MA).
2. PBS powder for reconstitution in 1 L distilled water (VWR International, cat. no. 429942P); pH should be adjusted to 7.2 after reconstitution.
3. Paraformaldehyde (VWR International, cat. no. 28794295): for preparation of 4% (w/v) formaldehyde, add 4 g paraformaldehyde powder in 100 mL 1X PBS buffer. Stir the solution with a magnetic stirrer at 70°C until all the powder is dissolved. The prepared solution can be stored at –20°C or use immediately. Bring to room temperature before use.
4. Sodium azide (VWR International, cat. no. 103692K).
5. Rat tails.
6. Glacial acetic acid (Sigma-Aldrich, cat. no. A 6283).
7. Cell scraper (Orange Scientific, cat. no. 2060100).
8. Medium 199 (Sigma-Aldrich, cat. no. M 9163).
9. Embryo dish (VWR International, cat. no. 406020401).
10. Ethanol 99.7–100% v/v, GPR (VWR International, cat. no. 28304 7K).
11. Xylene low in sulfur GPR (VWR International, cat. no. 305756G).
12. Trisodium citrate dihydrate (VWR International, cat. no. 007191174).
13. Trypsin (DakoCytomation, Cambridgeshire, UK; cat. no. S2012).
14. Trypsinization buffer: 50 m*M* Tris-HCl, 150 m*M* NaCl, and 0.1% $CaCl_2$ at pH 7.8.
15. Tris-HCl (VWR International, cat. no. 10315 4M).
16. NaCl (VWR International, cat. no. 10241 4J).
17. $CaCl_2$ (VWR International, cat. no. 10070 3H).
18. Rabbit antihuman prosurfactant protein C (proSPC) polyclonal antibody (Chemicon Europe, cat. no. AB3786).
19. Antirabbit immunoglobulin G (whole molecule)–fluorescein isothiocyanate antibody produced in goat (Sigma-Aldrich, cat. no. F9887).
20. Normal goat serum (Sigma-Aldrich, cat. no. G9023).
21. Antibody diluent with background-reducing components (DakoCytomation, cat. no. S 3022).
22. Vectashield mounting medium containing propidium iodide (Vector Laboratories Inc., Burlingame, CA; cat. no. H-1300).

3. Methods

3.1. Tissue Culture

3.1.1. Gelatin Coating of Culture Surfaces

1. Add an adequate volume of 0.1% gelatin solution to the culture surface to cover the entire surface and leave in a 37°C incubator for at least 1 h.
2. Aspirate the gelatin solution before use and add the cell suspension.

3.1.2. Thawing Cells

1. Prepare the culture plate and warm the water bath to 37°C; prepare culture medium (supplemented high-glucose DMEM) and warm it to 37°C.
2. Remove the frozen cell vial from the liquid nitrogen, submerge the vial in the water bath, and check for thawing.
3. Remove the vial just before the final ice crystal disappears and wipe the outside of the vial with 70% ethanol.
4. Transfer cells to a centrifuge tube and add 9 mL medium dropwise, swirling constantly.
5. Centrifuge the cells for 5 min at 200*g* (*see* **Note 2**).
6. Aspirate medium from the pellet, resuspend cells in the medium, and apply to the gelatin-coated plate.

3.1.3. Cell Splitting (Trypsinization)

1. Feed cells about 1 h before splitting.
2. Warm an appropriate amount of trypsin-EDTA solution and culture medium.
3. Aspirate medium from cultured cells and wash three times with 1X PBS.
4. Add trypsin-EDTA solution and put cells in a 37°C incubator for 5–10 min.
5. Gently pipet the cells up and down to separate them into single cells.
6. Transfer the cells into a centrifuge tube and add culture medium to the amount of splitting medium (*see* **Note 3**).
7. Centrifuge the cells for 5 min at 200*g*.
8. Remove the medium. Resuspend and plate out the cells as required.

3.1.4. Cell Freezing

This freezing protocol is applicable to both murine ES cells and STO/SNL cell lines.

1. Put freezing medium on ice.
2. Trypsinize the cells as described in **Subheading 3.1.3.**
3. Add culture medium to the splitting medium and centrifuge the cells at 200*g* for 5 min.
4. Remove the medium and resuspend cells in an appropriate volume of medium.
5. Count the cells and centrifuge as before.
6. Remove the medium and add an appropriate volume of the freezing medium so that the cell concentration is between 2×10^6 and 5×10^6 per milliliter of suspension.
7. Aliquot suspension into 1-mL cryovials, transfer the vials into a 100% isopropyl alcohol bath, and leave the bath in the –70°C freezer overnight. Alternatively, put them in a –20°C freezer for 1 h, then transfer to a –70°C freezer overnight.
8. Transfer to liquid nitrogen the next day.

3.1.5. Culture of Murine Fibroblast Feeder Cell Line

STO/SNL feeder cells are a derivative of the standard STO cell lines (murine embryonic fibroblasts). They are transformed with a LIF-producing plasmid ***(36)*** containing a neo-resistant (neor) gene. These cells are grown on gelatin B-coated tissue culture flasks (*see* **Subheading 3.1.1.**) in inactivation medium (*see* **Subheading 2.1.1., item 3**) plus 200 µg/mL geneticin (G418) sulfate.

1. Thaw a frozen cell vial (*see* **Subheading 3.1.2.**) and resuspend cells in STO/SNL cell medium.
2. Seed onto a gelatin-coated tissue culture flask.
3. Change the medium every other day.

4. When the cells reach confluence, they could be split (for propagation) or inactivated (for use as a feeder layer for ES cells; *see* **Subheading 3.1.4.**).

3.1.6. Inactivation of Fibroblasts

Inactivated fibroblasts are grown on gelatin B-coated tissue culture flasks in supplemented high-glucose DMEM (without geneticin).

1. Aspirate culture medium from the cells and replace with 1X mitomycin C solution.
2. Incubate for 2–3 h.
3. Wash three times with PBS (without Ca^{2+} and Mg^{2+}) and trypsinize the cells (*see* **Subheading 3.1.3**).
4. Freeze the cells or plate out as required.

3.1.7. Culture of Murine ES Cells

3.1.7.1. Culture of Undifferentiated Cells

1. Seed mitotically inactivated STO/SNL cells in gelatinized tissue culture flasks 24 h before seeding of ES cells in supplemented high-glucose DMEM (excluding G418).
2. Seed ES cells in the same medium and change the medium every other day (*see* **Note 4**).
3. When a subconfluent dish of ES cells is obtained, the cells are weaned off the feeder layer by splitting twice without adding any new feeder cells to the subsequent cultures but adding 1000 U/mL LIF (ESGRO).

3.1.7.2. Formation of EBs

1. Perform limited trypsin digestion (0.05% trypsin/0.53 m*M* EDTA in 0.1 *M* PBS without calcium or magnesium) on confluent cultures of undifferentiated ES cells to produce clusters of 8–15 cells.
2. Add the culture medium to the amount of the splitting medium (*see* **Note 3**). The culture medium is the same medium used for the culture of undifferentiated cells but without LIF.
3. Transfer the cells onto Nalgene polymethylpentene Petri dishes and feed the cells every other day.
4. To change the medium, collect the contents of the Petri dish into a conical centrifuge tube and allow the EBs to settle for a few minutes (do not centrifuge).
5. Aspirate the supernatant medium and replace it with fresh medium.
6. Transfer the entire contents into the Petri dish. The EBs are kept in nonadherent conditions for 10 d.

3.1.7.3. Post-EB Culture

1. Transfer 10-d-old EBs into nongelatin-coated tissue culture flasks.
2. Feed the cells every other day with the same medium described for EBs.
3. Maintain the cells in this culture condition for 20 d without subculturing (*see* **Note 5**).
4. Change the culture medium on d 21 to SAGM and maintain the cells in this medium for 3 d. Medium is changed every day.
5. Harvest cells after this culture period.

3.2. Reverse Transcriptase Polymerase Chain Reaction

3.2.1. Total RNA Isolation by the Silica Surface

Total RNA isolation by the silica surface has been integrated into the SV Total RNA Isolation System kit. The procedure involves a DNase digestion step as well. All the

data below are adopted from Promega's instructions for use of the Z3100 and Z3101 products.

1. Harvest the cells by trypsinization as described in **Subheading 3.1.3.**, neutralize the trypsin with serum-containing medium, and count the cells.
2. Centrifuge and discard the supernatant.
3. Wash the pellet with the ice-cold PBS and aliquot the cells into $< 5 \times 10^6$.
4. Centrifuge and discard the supernatant.
5. Add 175 µL lysis buffer for each 5×10^6 aliquot.
6. Mix by pipetting or vortexing (*see* **Note 6**).
7. Pass the lysate through a 20-gage needle four or five times and expel it into a 1.5-mL tube.
8. Add 350 µL dilution buffer and mix by inverting three or four times.
9. Incubate at 70°C for 3 min and centrifuge at 12,000–14,000*g* for 10 min.
10. Take the supernatant and transfer to another Eppendorf tube. Do not disturb the pellet.
11. Add 200 µL 95% ethanol into the transferred supernatant and mix by pipetting.
12. Transfer the mixture to the spin basket (containing silica membrane) and mount it on the collection tube.
13. Centrifuge at 12,000–14,000*g* for 1 min.
14. Discard the elute and put the spin basket back into the collection tube.
15. Add 600 µL wash solution and centrifuge at 12,000–14,000*g* for 1 min.
16. Empty the collection tube and put the basket back.
17. Prepare DNase incubation mix as below (keep the order) and mix gently by pipetting; these measurements are for one sample: 40 µL yellow core buffer, 5 µL 0.09 *M* $MnCl_2$, and 5 µL DNase I.
18. Apply 50 µL DNase incubation mix to the silica membrane and spread over all the membrane.
19. Incubate at room temperature for 15 min and add 200 µL DNase stop solution.
20. Centrifuge at 12,000–14,000*g* for 1 min.
21. Add 600 µL wash solution.
22. Centrifuge at 12,000–14,000*g* for 1 min and empty the collection tube.
23. Add 250 µL wash solution and centrifuge at high speed for 2 min.
24. Remove one elution tube from the tray and transfer the spin basket from the collection tube to the elution tube.
25. Add 100 µL nuclease-free water, and spread it to all the membrane, and centrifuge at 12,000–14,000*g* for 1 min.
26. Discard the spin basket and put the RNA on ice.
27. Quantitate the RNA by spectrophotometry (*see* **Note 7**) and determine its quality by RNA denaturing agarose gel electrophoresis.
28. Store the RNA at –70°C after quantification.

3.2.2. Complementary DNA Synthesis by an MMLV-Derived RTase

The MMLV-derived RTase in the MessageSensor RT kit (M-MLV RT) is an RNase H-plus form of the RTase enzyme, so this obviates the need for a separate RNase H treatment step.

1. Add 1 µg RNA sample in a PCR tube and adjust the volume to 10 µL by RNase-free water.
2. Prepare the master mix by adding 2 µL 10X RT-PCR buffer, 4 µL 2.5 m*M* dNTP mix, 2 µL 50 µ*M* random primer, 10 U RNase inhibitor, and 1 µL M-MLV-RT.
3. Add the master mix (10 µL) to the RNA sample.
4. Incubate at 50°C for 30 min followed by 95°C for 10 min.

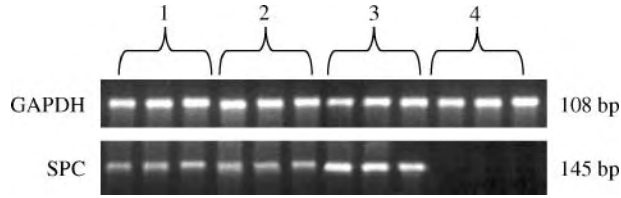

Fig. 1. DNA agarose gel electrophoresis of real time reverse transcriptase polymerase chain reaction (RT-PCR) products. The amplicons sizes are as expected. RT-PCR of each sample was performed in triplicate. Treatment of samples were as follows: 1, supplemented DMEM; 2, SAGM; 3, RNA from mouse lung; 4, RNA from STO/SNL cell line. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

3.2.3. First-Strand Complementary DNA Precipitation

The protocol for first-strand cDNA precipitation was developed by Liss *(37)* (*see* **Note 8**).

1. Add 1 µg glycogen, 250 ng poly(C) RNA, 250 ng of poly(dc) DNA, and a 0.1 vol 2 *M* sodium acetate at pH 4.5 to 20 µL RT reaction.
2. Precipitate cDNA overnight with 3.5 vol of 100% ethanol at –20°C.
3. Centrifuge at 13,000*g* for 60 min at 4°C and discard the supernatant.
4. Add 100 µL 75% ethanol and centrifuge at 13,000*g* for 15 min at 4°C.
5. Discard the supernatant and dry the cDNA pellet in the thermal heating block at 45°C until all ethanol is evaporated.
6. Dissolve the cDNA in an appropriate volume of RT-PCR-grade water by incubating the sample for 60 min at 45°C.
7. Quantitate the cDNA using a spectrophotometer.

3.2.4. Real-Time PCR

1. Prepare enough master mix to be distributed between your samples (*see* **Note 9**) (the data presented here are adopted from AB Applied Biosystem's protocol for the use of SYBR green PCR and RT-PCR reagents). For a 50-µL reaction, add the following: 5.0 µL 10X SYBR green PCR buffer, 6.0 µL 25 m*M* $MgCl_2$, 4.0 µL dNTP mix, 0.25 µL AmpliTaq Gold, and 0.50 µL AmpErase UNG. Add 300 ng cDNA template and increase the volume to 50 µL with RT-PCR-grade water.
2. Adjust the temperature profile as follows: 50°C for 2 min, followed by 94°C for 10 min and 50 cycles of template denaturation at 95°C (15 s), primer annealing, and extension at 60°C (1 min).
3. Normalize the expression of SPC gene to that of glyceraldehyde-3-phosphate dehydrogenase (*see* **Note 10**).
4. Use relative standard curve method for calculation of relative expression of SPC gene in different samples.

The results of this experiment showed that SAGM increases the mRNA expression level of SPC by a factor of 8 when compared to that of supplemented high-glucose DMEM ($p < 0.05$, *t*-test). **Figure 1** shows the DNA gel electrophoresis of the PCR products.

3.3. Immunocytochemistry

3.3.1. Fixation

1. Bring 4% formaldehyde to room temperature.
2. Discard the cell culture medium and add enough 4% formaldehyde to the culture surface to cover all the area.
3. Leave for 30 min at room temperature.
4. Wash three times with 1X PBS.
5. Cover the culture surface with 0.01% (w/v) sodium azide (*see* **Note 11**) in PBS and store at 4°C until further processing.

3.3.2. Embedding

3.3.2.1. Rat Tail Collagen

For easy handling of the tissue, it is embedded in rat tail collagen. Rat tail collagen (mainly type I collagen) can be provided by Bornstein's method *(46)*:

1. Remove the rat tail tendon fibers.
2. Add these fibers to glacial acetic acid:water (1:1000) solution; 1 g tendon is added to 150 mL acetic acid solution.
3. Leave the fibers in the solution on a magnetic stirrer at 4°C for 48 h.
4. Centrifuge for 20 min at 200*g*.
5. Discard the pellets.

3.3.2.2. Embedding in Collagen

1. Scrape the tissue from the culture surface using a cell scraper (*see* **Note 12**).
2. Place 1.8 mL rat tail collagen in prechilled 50-mL polypropylene tube and add 0.2 mL 10X medium 199 and mix.
3. Add an adequate volume of 0.35 *M* NaOH. The color of the mixture should change from yellow to orange-red but not too red or the gel will not form properly. About 98 µL gives the best color in this volume.
4. Transfer the solution to ice immediately.
5. Completely surround the tissue with a small volume of the collagen solution and allow to gel at room temperature for about 20–30 min.

3.3.2.3. Embedding in Paraffin

1. For embedding in paraffin, the sample should be dehydrated first. Place gel-embedded tissue in an embryo dish with 1X PBS and allow it to sit for 15 min.
2. Remove half of the volume of PBS in the dish and add a half volume of 50% ethanol.
3. Perform this step three times over a 30-min period.
4. Remove half of the volume of 50% ethanol and add a half volume of 70% ethanol.
5. Perform this step three times over a 30-min period.
6. Remove half of the volume of 70% ethanol and add a half volume of 90% ethanol.
7. Perform this step three times over a 30-min period.
8. Remove half of the volume of 90% ethanol and add a half volume of 95% ethanol.
9. Perform this step three times over a 30-min period.
10. Remove half of the volume of 95% ethanol and add a half volume of 100% ethanol.
11. Perform this step twice over a 20-min period.

12. Remove all of the ethanol in the dish and replace with 100% ethanol.
13. Perform this step three times over a 30-min period (*see* **Note 13**).
14. Remove half of the volume of ethanol and add a half volume of xylene.
15. Perform this step four times over a 1-h period. The collagen gel should lose its opacity and become clear.
16. Remove half of the volume of xylene and add a half volume of paraffin-saturated xylene.
17. Prepare a saturated solution of paraffin in xylene prior to this step.
18. Leave at room temperature overnight.
19. Turn on the oven so that it is at the right temperature (heated to 60°C) for the following day.
20. Place beaker of paraffin in the oven so that it can melt overnight.
21. The next day, place dishes in the oven for 20 min.
22. Pour out half of the liquid in the dish carefully.
23. Replace the decanted liquid with molten paraffin.
24. Place in the oven for 30 min and repeat the previous step.
25. Pouring out of half of the liquid and its replacement with molten paraffin should be repeated three times.
26. Fill half of the embedding mold with molten paraffin and place it on top of a hot plate (60°C). Use a warm needle to remove tissue/gel from the embryo dish and carefully place in the mold.
27. Position the tissue/gel appropriately.
28. Place mold on cold surface for 1 min.
29. Fill remainder of mold with molten paraffin.
30. Place paraffin-embedded tissue in refrigerator to cool and harden.
31. Cut and mount sample on a glass slide after hardening.

3.3.3. Staining

After cutting and mounting on the slide, the sample should be rehydrated (dewaxed) and then undergo the staining procedure.

3.3.3.1. Dewaxing

Incubate the glass slides in the following solutions one after the other:

1. 100% xylene for at least 5 min.
2. 100% xylene for at least 5 min.
3. 100% ethanol for at least 3 min.
4. 100% ethanol for at least 3 min.
5. 70% ethanol for at least 3 min.
6. Distilled water.

3.3.3.2. Antigen Retrieval

1. Autoclave twice in 0.294% (w/v) trisodium citrate in distilled water (pH 6.0).
2. Let the solution and slides cool and then put the slides in the distilled water.
3. Dilute trypsin to 1X by trypsinization buffer.
4. Trypsinize at room temperature for 60 s.

3.3.3.3. Staining Procedure

1. Incubate samples in 5% (v/v) normal goat serum in 1X PBS for 20 min to block the hydrophobic binding sites in the samples.

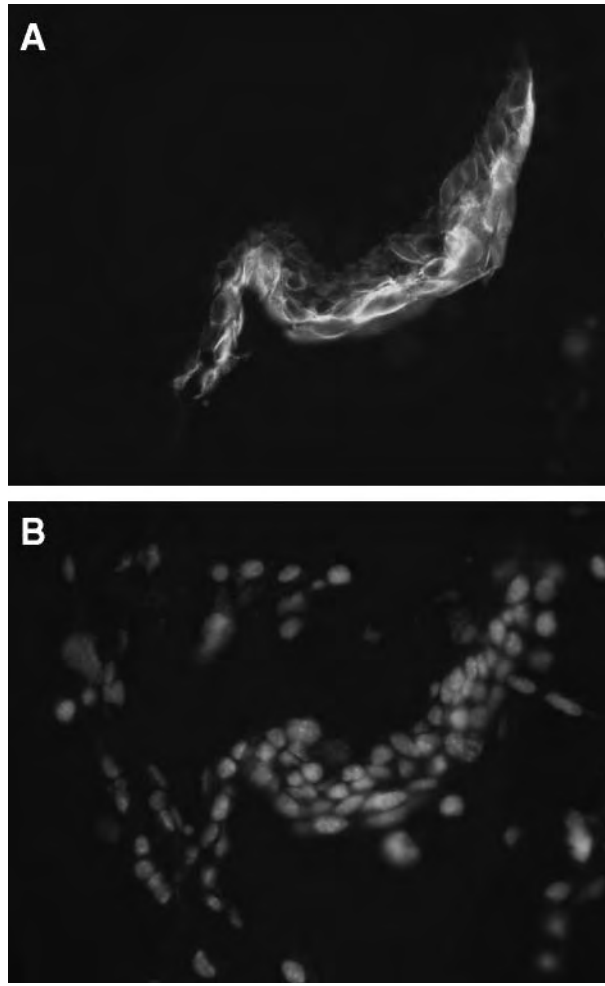

Fig. 2. (**A**) Immunofluorescent staining for prosurfactant protein C showing a cluster of positive cells (×40). (**B**) Same view as **A** counterstained for nuclei with propidium iodide (×40).

2. Dilute rabbit proSPC antibodies (primary antibodies) with antibody diluent with background-reducing components to 1/500.
3. Incubate samples in primary antibody at 4°C overnight.
4. Incubate samples in 1/100 dilution of fluorescein isothiocyanate-conjugated goat antirabbit immunoglobulin G for 1 h at room temperature.
5. Perform nuclear staining by Vectashield mounting medium containing propidium iodide.
6. View preparations under a fluorescent microscope at excitation wavelengths of 490 (fluorescein) and 545 (propidium iodide) nm.

Immunostaining of samples treated with SAGM and supplemented DMEM detected positive cells in clusters (**Fig. 2**), confirming translation of mRNA into protein. Cell

counting revealed that proSPC-expressing cells comprised 2.8 ± 0.23% of the total cell population in SAGM-treated samples and 0.5 ± 0.1% in supplemented DMEM-treated samples ($p < 0.05$, χ^2 test).

4. Notes

1. 2-ME loses its thiol (–SH) group in the presence of metallic salts within 1–2 d. Therefore, 2-ME must be added fresh to the culture medium immediately before use.
2. The duration and centrifugal force may differ for different cell lines, but generally 5 min at 200*g* is suitable.
3. The serum in the culture medium inhibits trypsin.
4. When undifferentiated cells are thawed from the frozen state, in the presence of feeder cells they grow faster, and their recovery rate is higher than when cultured in the feeder-free condition.
5. The protocol presented here for the post-EB culture involves culture of the cells for 20 d without splitting. Culture with splitting once weekly has also been tried, but when the cells are split once weekly, a significant number of cells die after adding the serum-free medium SAGM. As the aim of this study was comparison of SAGM with supplemented high-glucose medium and this cell loss caused sampling bias, we had to change our protocol as presented here. Further experiments are needed to show whether the change of medium to SAGM in the protocol with once-weekly splitting of the cells leads to selective loss of nonpneumocyte type II cells or leads to nonselective cell loss.
6. In the total RNA isolation protocol, the samples may be stored at –20 or –70°C after homogenization in lysis buffer.
7. In spectrophotometry, nucleic acids absorb ultraviolet radiation at 260 nm, and proteins absorb at 280 nm. Pure RNA will exhibit an A_{260}/A_{280} ratio of 1.7–2.1. Low ratios are typically caused by protein contamination. Dilution of RNA samples for spectrophotometry is done by TE buffer at pH 7.0. Organic substances such as phenol, guanidine, carbohydrates, peptides, or aromatic compounds absorb ultraviolet radiation at 230 nm. Pure RNA will exhibit an A_{260}/A_{230} ratio of 1.8–2.2. Low ratios are typically caused by contamination with organic substances such as guanidine or phenol. This interferes with downstream processing. Therefore, a decontamination procedure should be performed. Absorbance at A_{320} should be zero or less than 5% of that at A_{260}. Values greater than this indicate the presence of particulates in the solution or dirty cuvettes and result in erroneous readings. Measurement of nucleic acid concentration is only reliable when the A_{260} is less than 0.1. This value is independent of the measuring device and is based on the disturbing influence of impurities and particles on the measurement result, which is especially high at A less than 0.1. Most accurate readings are usually obtained in A_{260} of 0.5–1.5 (personal communication with Eppendorf Application Hotline).
8. As the expression of SPC mRNA level was low, the amount of template in the real-time RT-PCR experiment had to be increased. This increased the concentration of RT reaction mixture in the final real-time PCR reaction and caused a considerable distortion of the PCR amplification reaction, presumably by inhibiting *Taq* DNA polymerase *(47,48)*. Therefore, we had to precipitate the cDNA from our RT reaction.
9. Always calculate and prepare for at least 10% more than needed because of losses of PCR mix by pipetting.
10. The number of cycles required by the SYBR green I dye fluorescence to become significantly higher than background fluorescence in the log phase of amplification reaction is used as a measure of abundance.

11. Sodium azide prevents contamination of the sample with microorganisms.
12. For immunocytochemistry, we have tried staining of the cells grown on the chambered slides without scraping and embedding in paraffin. The results showed clusters of overlapping positive cells. Overlapping of the cells made cell counting inaccurate. Therefore, for the purpose of counting, we embedded the cells in paraffin and cut them.
13. It is very important that no water is left in the sample before the addition of xylene.

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17

Derivation and Characterization of Thyrocyte-Like Cells From Embryonic Stem Cells In Vitro

Reigh-Yi Lin and Terry F. Davies

Summary

The thyroid gland is centrally important in metabolic homeostasis, growth, and development. Defects in any of the multiple steps required for normal thyroid development and thyroid hormone synthesis have been shown to result in thyroid-dependent pathology in a variety of animal models of disease and in humans. However, there are many unanswered questions regarding the cellular and molecular mechanisms leading to thyrocyte abnormalities. A lack of knowledge regarding thyroid stem/progenitor cell differentiation and proliferation has further hampered attempts to gain more insight into thyroid-associated disease mechanisms. In this chapter, we present a culture system that has successfully generated thyrocyte-like cells from undifferentiated embryonic stem cells in vitro. Access to specific early-developing cell populations, such as the thyroid stem cells in the embryonic stem cell system, provides an experimental approach to characterizing key molecular events involved in thyrocyte commitment.

Key Words: Embryonic stem cells; thyroid; thyroid-stimulating hormone; thyroglobulin.

1. Introduction

Thyroid stem cell development is poorly understood. The development of in vitro models, which reproduce the structural and functional characteristics of thyroid cells in vivo, is important for understanding thyroid cell biology and the thyroid diseases. Such models are currently not available. Existing approaches to investigate thyroid cell proliferation and differentiation rely on the in vitro culture of mouse or human thyroid cells *(1–6)* or established cell lines of rat (FRTL-5, PCCL-3, and WRT) *(7,8)* or more recently human thyroid *(6)*.

Although these approaches have provided useful information, each has its limitations. Primary cell cultures are contaminated with other cell types, difficult to standardize, and show minimal evidence of follicular cell proliferation. The widely used FRTL-5 cells, which originated from adult rat thyroid glands, can be propagated indefinitely and retain

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most of the features of differentiated thyrocytes, such as the ability to respond to thyroid-stimulating hormone (TSH) by cyclic adenosine 5′-monophosphate (cAMP) production, iodide trapping, and thyroglobulin (Tg) secretion *(4,9)*. However, FRTL-5 cells have been shown to be tetraploid rather than diploid *(10)*. With age, the characteristics of FRTL-5 cells in culture can change, leading to poor differentiation and accelerated growth *(11)*. Furthermore, they are incapable of forming follicles in culture, have lost some of the basic control mechanisms of the cell cycle, and when injected into nude mice, some FRTL-5 cell clones develop TSH-dependent tumors *(10)*.

In an attempt to establish a better model to study early thyroid cell differentiation, we developed a novel embryonic stem (ES) cell-based approach. ES cells are continuously growing cell lines isolated from the inner cell mass of the blastocyst *(12)* that can be propagated indefinitely in an undifferentiated state with leukemia inhibitory factor (LIF) *(13,14)*. When ES cells are induced to differentiate in vitro in the absence of LIF, they form three-dimensional embryo-like structures termed embryoid bodies (EBs) that are composed of derivatives of the three primary germ layers and have the potential to differentiate into cells of all lineages *(15)*. The EB system recapitulates stages of early embryogenesis through gastrulation with formation of postimplantation embryonic tissues *(15)*. Using the ES/EB differentiation model, ES cell-derived hematopoietic precursors *(14,16)*, neural precursors *(17–19)*, insulin producing β-cells *(20,21)*, hepatocytes *(22)*, and cardiomyocytes *(23)* have been characterized and transplanted into recipient animals.

ES cells have extraordinary self-renewal and differentiation capacities and thus offer several advantages over other models to study thyroid cell differentiation. First, ES cell self-renewal can be perpetuated over many generations, resulting in considerable amplification of cell numbers, thereby providing an unlimited source of thyroid cells for genetic manipulation and analysis. Second, access to thyroid stem/progenitor cell populations in EBs will enable detailed molecular analysis of the development of this lineage and may provide a molecular explanation for birth defects and diseases such as congenital hypothyroidism and thyroid cancer that involve aberrant cell proliferation and differentiation. Third, ES cells may provide a model system for evaluating genetic changes resulting in embryonic lethality and thus are precluded from in vivo analysis.

The thyroid gland consists of several cell types that are derived from all three embryonic germ layers. Thyroid follicular cells, which represent the most abundant cellular populations in the gland, are of endodermal origin. The thyroid gland is the only tissue in the body that can absorb iodine and convert it into thyroid hormones (triiodothyronine [T_3] and thyroxine [T_4]). The gland is centrally important in metabolic homeostasis, growth, and development. A number of markers for thyroid follicular cells have been identified. Three transcription factors (TTF1, TTF2, and Pax8) have been implicated in the control of transcription of Tg and thyroperoxidase (TPO) genes throughout thyroid development *(24–27)*. Four markers of thyroid differentiation, including the Na^+/I^- symporter (NIS), TPO, Tg, and TSH receptor (TSHR), dictate the complex machinery of thyroid hormone synthesis and can be considered differentiation markers of thyroid follicular cells *(28–30)*.

We demonstrated that mouse ES cells gave rise to thyrocyte-like cells in vitro. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of the differentiating

EB cells revealed a temporal appearance of mRNA transcripts for a number of thyroid differentiation genes, including NIS, TPO, Tg, and TSHR *(31)*. Immunofluorescent analysis demonstrated the presence of TSHR-positive cells as outgrowths from EBs *(31)*. Accordingly, this area of cells also expressed Pax8 and another thyroid transcription factor, TTF2 *(31)*. Of importance, TSH, the main regulator of the thyroid gland, was able to maintain the expression of Pax8 and TSHR genes during EB differentiation *(31)*. Furthermore, thyroid-specific function, such as cAMP generation by TSH, was maintained in this model *(31)*.

Together, these results suggested that the developmental program associated with thyrocyte development can be recapitulated in the ES/EB model system. These observations provide a first step in exploring the potential of thyroid stem cells to use them as a model system to study the differentiation mechanisms underlying the thyrocyte lineage. In this chapter, we describe protocols for the culture conditions that cover the induction and specification of the thyroid cell lineage from differentiating ES cells.

2. Materials

All reagents and materials used in ES cell culture are sterile.

2.1. ES Cell Line

The ES cell line most commonly used in our laboratory is the CCE line *(32)*. The protocols described here are thus optimized for this cell line.

2.2. Cell Culture Media

1. High-glucose Dulbecco's modified Eagle's medium (DMEM) with L-glutamine (Gibco, Grand Island, NY; cat. no. 11965-092).
2. Iscove's modified Dulbecco's medium (IMDM) (Gibco; cat. no. 12440-053).

2.3. Growth of Embryonic Fibroblast Cells

1. IMDM-embryonic fibroblast (EF) medium: IMDM containing 10% fetal bovine serum (FBS) (StemCell Technologies Inc., Vancouver, Canada; cat. no. 06951) (*see* **Note 1**), 100 U/mL penicillin-streptomycin (Gibco; cat. no. 15070-063), and 1.5×10^{-4} *M* monothioglycerol (MTG) (Sigma, St. Louis, MO; cat. no. M-6145).
2. Trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco, cat. no. 25300-054): 0.05% trypsin and 0.53 m*M* EDTA-4Na.
3. EF wash buffer: 95% IMDM and 5% FBS.

2.4. Growth and Maintenance of ES Cells

1. DMEM-ES or IMDM-ES medium: DMEM or IMDM containing 15% FBS, 100 U/mL penicillin-streptomycin, 2 m*M* L-glutamine, 1.5×10^{-4} *M* MTG, and 10 ng/mL recombinant mouse LIF (StemCell Technologies, cat. no. 02740).
2. Trypsin-EDTA (Cellgro, Herndon, VA; cat. no. 25-053-CI): 0.25% trypsin and 0.1% EDTA.
3. L-Ascorbic acid (Sigma, cat. no. A-4544): prepare a stock solution of 5 mg/mL in cell culture-grade distilled water (Gibco, cat. no. 15230-162), sterile filter, aliquot, and store at –20°C. Use once and discard excess.
4. Gelatin (Sigma, cat. no. G-1890): prepare a 0.1% solution of gelatin in 1X phosphate-buffered saline (PBS), dissolve, and sterilize by autoclaving. Aliquot in 50-mL portions and store at 4°C.

5. Gelatinized flasks and dishes: add enough 0.1% gelatin solution to cover the surface of a dish or flask. Let the solution sit for at least 10 min at room temperature. Aspirate excess solution completely prior to use.
6. ES wash buffer: 95% DMEM and 5% FBS.

2.5. Induction of EBs

1. Knockout serum replacement medium (Knockout SR) (Gibco, cat. no. 10828) (*see* **Note 2**).
2. IMDM-FBS medium: IMDM containing 5% FBS and 1.5×10^{-4} *M* MTG.
3. Differentiation medium I: IMDM containing 15% FBS (Atlas Biological, Fort Collins, CO; cat. no. F-0500-D), 5% protein-free hybridoma medium II (Gibco, cat. no. 12040-077), 2 m*M* L-glutamine, 100 U/mL penicillin-streptomycin, 200 µg/mL transferrin (Boehringer Mannheim, Ridgefield, CT; cat. no. 652202), 0.5 mg/mL ascorbic acid, 200 µg/mL transferrin, and 1.5×10^{-4} *M* MTG (*see* **Note 3**).
4. Differentiation medium II: IMDM containing 15% Knockout SR, 5% protein-free hybridoma medium II, 2 m*M* L-glutamine, 100 U/mL penicillin-streptomycin, 200 µg/mL transferrin, 0.5 mg/mL ascorbic acid, 200 µg/mL transferrin, and 1.5×10^{-4} *M* MTG.
5. Bacterial Petri dish 60 × 15 mm (VWR International Inc., Bridgeport, NJ; cat. no. 25384-090) (*see* **Note 4**).
6. DNAse I (Calbiochem, San Diego, CA; cat. no. 260912): to prepare a stock solution, dissolve 10 mg DNAse I into 10 mL cell culture-grade distilled water. Aliquot in 1-mL amounts and store frozen at –20°C. Use aliquots once and discard excess.
7. Collagenase/DNAse: add DNAse at a final concentration of 10 µg/mL to collagenase (Sigma, cat. no. C-0130) prior to ES dissociation.

2.6. Differentiation of Thyroid Cells

1. Growth factor-reduced Matrigel (BD Biosciences, Bedford, MA; cat. no. 40230): thaw the stock bottle of Matrigel slowly on ice, dilute 1:1 with IMDM, aliquot in 0.5-mL amount, and store frozen at –20°C.
2. Human recombinant TSH (Sigma, cat. no. T-8931).
3. Differentiation medium III: IMDM containing 15% Knockout SR, 2 m*M* L-glutamine, 100 U/mL penicillin-streptomycin, 0.5 mg/mL ascorbic acid, 200 µg/mL transferrin, 1.5×10^{-4} *M* MTG, and 100 µU TSH.

2.7. RT-PCR Detection of Differentiation Markers

1. RNeasy minikit (Qiagen, Valencia, CA; cat. no. 74104).
2. Thermoscript RT-PCR system (Invitrogen, Carlsbad, CA; cat. no. 11146-024).
3. PCR buffer: 20 m*M* Tris-HCl at pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* of each dNTP, 0.2 m*M* of each primer, and 1 U *Taq* polymerase (Gibco, cat. no. 18038-042).
4. Molecular weight markers: 100-bp DNA ladder (Gibco, cat. no. 15628-019).

2.8. Immunofluorescence Analysis

1. Culture slides (BD Falcon, Franklin Lakes, NJ; cat. no. 0877425): four-chamber polystyrene vessel tissue culture-treated glass slide.
2. 4% paraformaldehyde in phosphate buffer: dissolve 4 g paraformaldehyde in 100 mL 0.2 *M* phosphate buffer. Heat to 60°C. Stir and clear the solution by adding 200 µL 5 *M* NaOH. Filter through Whatman no. 1 paper and store in aliquots at –20°C (*see* **Note 5**).
3. PBS-Tween (PBS-T) blocking buffer: 0.1% Tween-20, 1 mg/mL bovine serum albumin (BSA) in PBS.

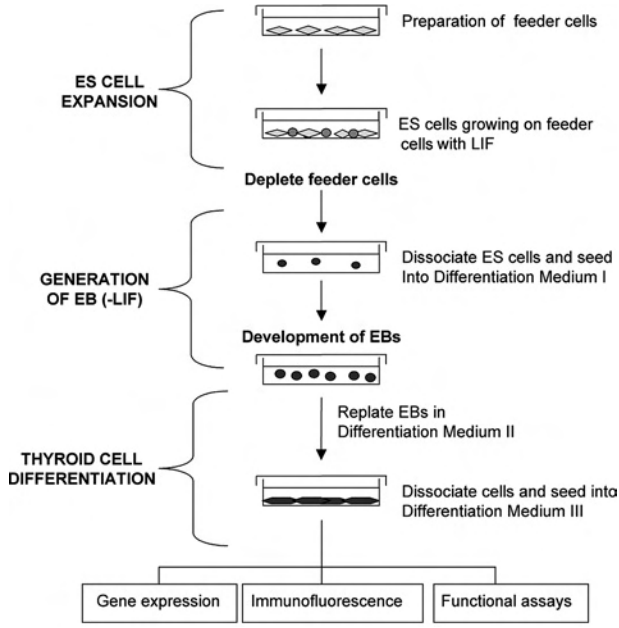

Fig. 1. Protocol for the generation of thyroid cells from embryonic stem cells (*see* text).

4. Vectashield mounting medium for fluorescence with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc., Burlingame, CA; cat. no. H-1200).
5. Primary antibodies (partial list): hamster antihuman TSHR ***(31)*** at 1:500; rabbit antirat TTF2 ***(31)*** at 1:100; rabbit antimouse Pax8 ***(31)*** at 1:500; rabbit antirat NIS ***(31)*** at 1:100; and rabbit antihuman Tg (DAKO, Carpinteria, CA; cat. no. A0251) at 1:1000.
6. Secondary antibodies (partial list): Alexa Fluor 594 goat antihamster immunoglobulin (Ig) G (Molecular Probe, Eugene, OR; cat. no. A-21113) at 1:2000; Alexa Fluor 594 chicken antirabbit IgG (Molecular Probe, cat. no. A-21442) at 1:2000; and Alexa Fluor 488 chicken antirabbit IgG (Molecular Probe, cat. no. A-21441) at 1:2000.
7. Dilution buffer for all antibodies: 3% BSA and 0.1% NaN_3 in PBS.

2.9. Intracellular cAMP Assay

For the intracellular cAMP assay, we used cAMP Enzymeimmunoassay Biotrak (EIA) System (Amersham Biosciences, Piscataway, NJ; cat. no. RPN225).

3. Methods

The procedure for the generation of thyroid cells from ES cells involves three different stages: (1) ES cell expansion, (2) generation of EBs from ES cells, and (3) the differentiation of EB cells into the thyroid cell lineage. The method is outlined in **Fig. 1**.

3.1. Preparation of ES Cells for Differentiation

ES cells for in vitro differentiation are routinely maintained on γ-irradiated mouse (EFs) in medium containing LIF.

3.1.1. Thawing and Maintenance of EF Cells

1. Thaw a frozen vial of irradiated EF cells in a 37°C water bath with gentle agitation (*see* **Note 6**).
2. Sterilize the outside of the vial with 95% ethanol.
3. Transfer cell suspension into a 15-mL Falcon tube containing 3 mL EF wash buffer.
4. Spin down cells at 300*g* for 5 min at room temperature.
5. Resuspend cells in about 3 mL IMDM-EF medium.
6. Seed 5×10^5 cells into each well of a six-well plate. Incubate at 37°C incubator with 5% CO_2 and 95% humidity. When the cells reach confluency, they are ready to use for the growth of ES cells.

3.1.2. Growth and Maintenance of ES Cells

ES cells divided rapidly; therefore, cell cultures should be monitored daily and the cells passaged every 2 d.

1. Thaw frozen vial of ES cells at 37°C in a water bath with gentle agitation.
2. Transfer cell suspension into a 15-mL Falcon tube containing 3 mL ES wash buffer.
3. Spin down cells at 300*g* for 5 min at room temperature.
4. Resuspend cells in DMEM-ES medium.
5. Into each well of a six-well plate with confluent feeder cells, add 1×10^6 ES cells. It is important to plate ES cells as single cells; otherwise, ES cells will spontaneously differentiate even in the presence of LIF. Incubate at 37°C in a CO_2 incubator.
6. Grow the cells up to approx 80% confluency and then passage the cells (*see* **Note 7**).
7. To passage cells, aspirate the DMEM-ES medium, add 1.5 mL 0.25% trypsin-EDTA, and incubate at 37°C for 2 min. Dissociate the cells well by pipetting up and down. It is important to ensure that the dissociation is complete in less than 3 min. Add 3 mL DMEM-ES medium to dilute and inactivate the trypsin. Centrifuge cells at 300*g* for 5 min at room temperature.
8. Aspirate the supernatant and split the cells at a 1:5 ratio.
9. Maintain by regularly passaging cells every 2 d. Do not cultivate ES cells longer than 2 d without passaging, or the cells may differentiate spontaneously (*see* **Note 8**).

3.1.3. Depletion of Feeder Cells

1. Trypsinize the feeder and ES cells by centrifuging them at 300*g* for 5 min at room temperature.
2. Resuspend the cell pellet in an appropriate volume for cell counting. Seed the cells into a gelatinized flask in DMEM-ES medium. Culture for 2 d and passage into a gelatinized flask in DMEM-ES medium. The entire process of depleting feeder cells should not require more than three passages.

3.2. In Vitro Differentiation of ES Cells

For the development of ES cells into different cell lineages, cells may be induced to form three-dimensional aggregate EBs in the absence of LIF. We first initiate the induction of endoderm during EB differentiation. For endoderm development in an ES differentiation culture, exposure of EBs to serum for restricted periods of time is essential, as described previously *(33)*. In subsequent steps, EBs will be further induced to the thyrocyte lineage.

3.2.1. Induction of EBs

1. At 1 d prior to the initiation of EB formation, passage ES cells into IMDM-ES medium.
2. To remove ES cells from the dish, add 0.25% trypsin-EDTA. Incubate at 37°C for 2 min. Neutralize the trypsin with ES wash buffer and remove the cells from the dish by gently pipetting up and down at least three times.
3. Spin down cells at 300*g* for 5 min at room temperature.
4. Resuspend cells in IMDM-FBS and count the cells.
5. Seed ES cells at 5×10^3 cells in 60-mm bacteriological-grade Petri dishes (*see* **Note 9**).
6. Add 5 mL differentiation medium I (**Subheading 2.2.4.**) Swirl the Petri dish so the cells are fairly close together. Incubate at 37°C in a CO_2 incubator.
7. At 2 d after EB formation, collect EBs into a conical tube. Allow EBs to settle for 5 min by gravity to the bottom of the tube, then remove the supernatant with an aspirator. Add fresh differentiation medium II and transfer EBs to a new bacterial dish.
8. Repeat **step 8**. Change the medium during the differentiation period every second day.

3.2.2. Thyroid Cell Differentiation

Our studies showed that when EBs were plated onto Matrigel-coated dishes, they gave rise to thyroid progenitor cells that were capable of differentiating into thyroid cells. Exposure of EBs to serum replacement medium for restricted periods of time was essential for the establishment of this lineage. In addition, TSH can induce thyroid lineage formation in serum-free culture conditions. Here, we describe TSH-dependent thyroid lineage induction using a two-step culture protocol. In the first step, d 6 EBs are replated as a monolayer in gelatin-coated dishes in differentiation medium II for 7 d. For the second step, EB-derived cells are dissociated into single cells and cultured in a Matrigel-coated dish in differentiation medium III for an additional 5–7 d (*see* **Fig. 2**).

1. At d 6 after EB formation, transfer EBs to a 50-mL Falcon tube. Allow EBs to settle by gravity for approx 5 min. Aspirate medium.
2. Add appropriate volume of differentiation medium II to the cells.
3. Carefully transfer 5–10 EBs to gelatin-coated tissue culture dishes. Spread the EBs evenly in the dish. Incubate at 37°C in a CO_2 incubator.
4. Replace with fresh differentiation medium II every other day.
5. At 7 d after plating, add 0.25% trypsin-EDTA to cells, incubate them at 37°C for 2 min, and then stop the reaction with the addition of 1 mL FBS.
6. Dissociate cells by pipetting up and down five times and passage them through a 5-mL syringe with a 20-gage needle twice.
7. Spin down cells after addition of 5 mL IMDM wash buffer. Resuspend pellet in 10 mL collagenase/DNAse solution. Incubate at 37°C for 30 min. Gently mix the cells by swirling every 10 min. Vigorous agitation should be avoided.
8. Spin down cells and resuspend in differentiation medium III. Carry out a microscopic evaluation using trypan blue to check cell number and viability.
9. Seed cells in Matrigel-coated tissue culture dishes (*see* **Note 10**).
10. Change the medium every other day.
11. After 5–7 d in the differentiation medium III, thyroid cells are ready for genetic and functional analyses (*see* **Note 11**).

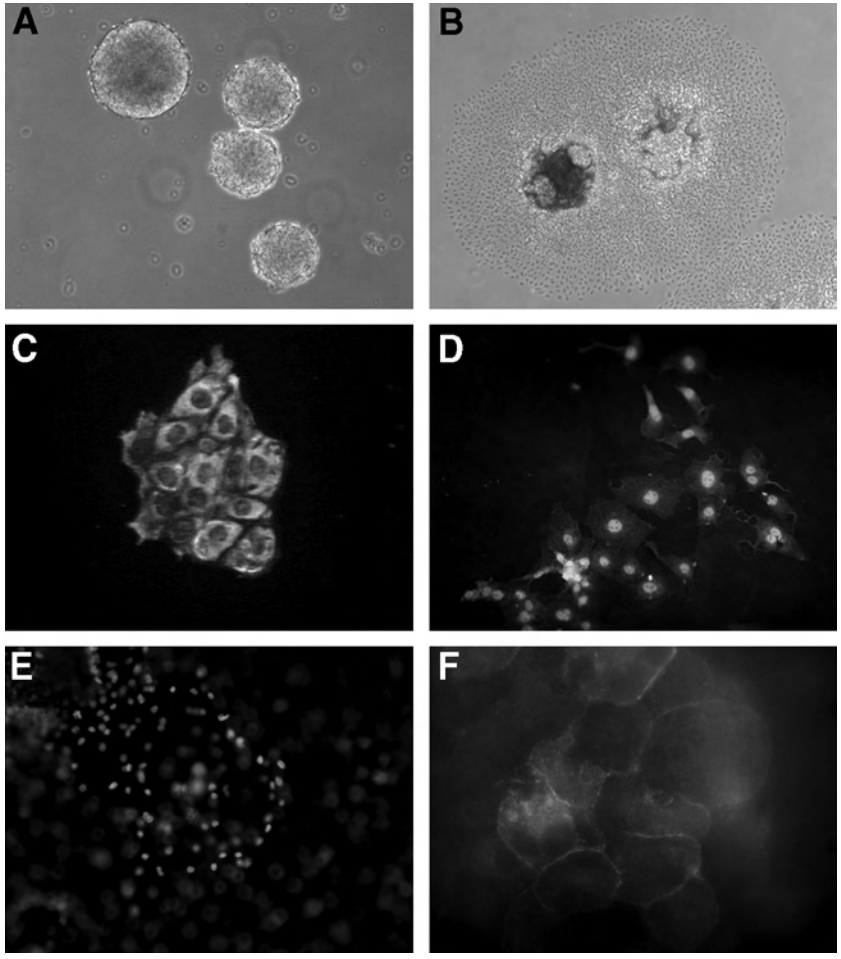

Fig. 2. Appearance of embryonic stem cell cultures. (**A**) Phase microscopy of growing embryoid bodies (EBs) in suspension after 4 d of differentiation. Variations exist in the appearance of the EBs. (**B**) Phase microscopy of EB-derived monolayer cells after 8 d of differentiation. (**C**) Immunofluorescent analyses of these cultures revealed that expression of the thyroid-stimulating hormone receptor was first detected after 8 d in some cells. (**D**) The Pax8 staining shown was a 10-d culture. It appeared that Pax8-positive cells were growing out from the spheres to form a monolayer of differentiated cells. Expression of Pax8 was seen in the nuclei of EB-derived cells. (**E–F**) Thyrocyte-like cell cluster on d 15 of differentiation. The cells had committed to further differentiation, as indicated by the unique three-dimensional structure and as demonstrated by the nuclear DAPI staining (**E**). (**F**) High magnification of anti-NIS immunofluorescence of the same field depicted in **E**. The staining revealed that NIS protein was detected at the plasma membrane (**F**).

Table 1
Primers Used and PCR Conditions

Gene	Forward primer	Reverse primer	Product size (bp)	Annealing temperature (°C)	Cycle number
NIS	GCTCTCATCAGCTA CCTAACTG	CTCAGAGGTTGGT CTCAACATC	243	56	35
Pax8	TGCCTTTCCCCAT-GCTGCCTCCGT GTA	GGTGGGTGGTGCG-CTTGGCCTTGAT GTAG	298	61	30
Tg	TGGGACGTGAAAG-GGGAATGGTGC	GTGAGCTTTTGGA-ATGGCAGGCGA	394	61	35
TPO	TGCCAACAGAAGC ATGGGCAAC	GCACAAAGTTCCC ATTGTCCAC	424	58	35
TSHR	GAGTGTGCGTCTC CACCATG	TTGCAGCCGCTGCA GAGTTGC	209	61	35
Oct4	GGCGTTCTCTTTG GAAAGGTGTTC	CTCGAACCACATCC TTCTCT	293	61	30
β-Actin	ATGAAGATCCTGA CCGAGCG	TACTTGCGCTCAGG AGGAGC	443	60	25

3.3. Characterization of ES Cell-Derived Thyrocytes

3.3.1. RT-PCR Detection of Differentiation Markers

Detection of the expression of thyroid genes can be achieved by RT-PCR. We have used a variety of thyroid gene markers to identify thyrocyte precursors and differentiated thyrocytes derived from ES cells.

1. Total RNA from four dishes per condition (undifferentiated ES cells, free-floating EB spheres, and differentiated cells growing from EBs) is prepared using the RNeasy kit and reverse transcribed into cDNA using the Thermoscript RT-PCR system according to the manufacturer's instructions.
2. PCR is performed using standard protocols with *Taq* polymerase. Amplification conditions are as follows: initial denaturation at 94°C for 30 s, annealing at 50–61°C for 45 s, extension at 72°C for 45 s, and final extension at 72°C for 7 min. Annealing temperature in all cases is set 2°C below the calculated denaturation temperature. **Table 1** gives the details of primer sequences, annealing temperature, cycle numbers, and the size of expected cDNA for each PCR reaction. The amount of cDNA into each sample is normalized using β-actin as a control.
3. Aliquots of the amplified PCR products are analyzed by electrophoresis in 2% agarose gels in Tris-borate-EDTA buffer and visualized by ethidium bromide staining.

3.3.2. Immunofluorescence Analysis

The formation of thyrocyte-restricted proteins in the EB outgrowths is analyzed by indirect immunofluorescent analysis.

1. Rinse four-well culture chamber slides containing EB outgrowths twice with PBS (*see* **Note 12**).
2. Fix cells with 4% paraformaldehyde in PBS at room temperature for 10 min.
3. Wash the cells twice with PBS.

4. Permeabilize the cells with 0.1% Triton X-100 in PBS for 10 min at room temperature. Wash the cells twice with PBS.
5. To prevent unspecific immunostaining, incubate the cells with 3% BSA in PBS or 2–5% serum of the same species in PBS as the secondary antibody.
6. Incubate with the primary antibody at 37°C in a humidified chamber for 60 min or at 4°C overnight.
7. Rinse the chamber slides with PBS-T three times at room temperature for 5 min.
8. Incubate with the fluorescent-labeled secondary antibody in a humidified chamber at 37°C for 45–60 min.
9. Rinse the chamber slides twice with PBS-T at room temperature for 5 min.
10. Remove the chambers according to manufacturer's instructions.
11. Embed the cover slips in Vectashield mounting medium with DAPI and analyze immunolabeled cells with a conventional fluorescent microscope.

3.3.3. Intracellular cAMP Measurement

Thyrocytes differentiated from ES cells express the TSHR. The TSHR belongs to a superfamily of G protein-coupled receptors that have a common structure consisting of seven transmembrane domains *(34)*. The TSHR transduces the TSH signal by promoting exchange of guanosine 5′-diphosphate to guanosine 5′-triphosphate on the α-subunit of G protein. The result is activation of the protein kinase A pathway mediated by adenylate cyclase and cAMP, which in turn regulates iodine uptake and transcription of Tg, TPO, and NIS mRNA, leading to thyroid hormone production *(34,35)*. Therefore, ES cell-derived thyrocytes are suitable for measurement of cAMP responses to TSH. The cAMP responses of cells are measured after incubating the cells for 1 h in the absence or presence of TSH using the cAMP enzyme immunoassay system.

1. Culture cells in standard 96-well microtiter plates (tissue culture grade) with cell concentrations between 10^4 and 10^5 cells/mL. Incubate the plate overnight at 37°C in a CO_2 incubator.
2. Aspirate medium. Add different concentrations of TSH (10–10^5 μU/mL) and incubate for 1 h before measurement.
3. Prepare assay buffer and lysis reagents and determine dose-dependent working standards (ranging from 12.5 to 3200 fmol) according to manufacturer's instructions.

4. Notes

1. Prescreen serum for the ability to maintain ES cells in the undifferentiated state. Select a batch that supports a good growth rate of ES cells. Pretested serum for ES cell growth is also available from several companies. It is recommended that FBS be thawed at room temperature or 4°C, mixed, and dispensed into 50-mL aliquots and frozen at –20°C. These aliquots can then be thawed as described and stored at 4°C for up to 1 mo.
2. Knockout SR contains no serum factors (i.e., complement). Heat inactivation is not required.
3. It is important to note that the serum lot selected for growth of ES cells is not always the best for EB generation. Serum added into the differentiation medium should be preselected based on its ability to support the efficient development of EBs and on the thyrocyte potential of the EBs that develop. To assess efficiency of EB development, total EB numbers from the differentiation cultures can be counted. Thyrocyte potential is determined by assaying EBs at different stages of development for the desired precursor populations.
4. It is important to test different brands of bacterial Petri dishes for lots that prevent sticking of the cell clumps, so that they will form EBs in suspension. We have found that VWR brand Petri

dishes (made in the United States) are the best dishes for the generation of EBs. In our experience, VWR brand Petri dishes allow the generation of EBs that rarely stick or aggregate.
5. Paraformaldehyde is toxic; make the solution in a fume hood.
6. Be careful not to immerse the vial above the level of the cap.
7. ES cells divide rapidly. Passage them when they reach a subconfluent state of 70–80%. We usually split ES cells at 1:5 ratio every other day. When passaging cells, cells should be trypsinized to a single-cell suspension as large clumps might differentiate even in the presence of LIF.
8. A long-term (longer than 2 wk) culture of ES cells can accumulate genetic abnormalities. It is important to use low-passage ES cells (<10 passages) for experiments.
9. It is also possible to seed at higher or lower densities and then culture the cells for a shorter or longer time.
10. Matrigel has been used to support the attachment and differentiation of thyroid cells. Thaw Matrigel as described in **Subheading 2.6.** Keep culture plates on ice; add diluted Matrigel to the vessel to be coated using cooled pipets. The amount of Matrigel should be sufficient to cover entire growth surface easily, usually 50 µL/cm^2 growth surface. Place plates at 37°C for 30 min. Plates are now ready to use.
11. In general, 5 d differentiation is enough to obtain differentiated thyroid cells. However, differentiation speed can vary depending on cell lines and other conditions. If thyrocyte differentiation is not enough, then a longer incubation period should be tried.
12. Culture chamber slides must be coated with Matrigel.

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18

Derivation and Characterization of Gut-Like Structures From Embryonic Stem Cells

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Summary

Embryonic stem (ES) cells have a pluripotent ability to differentiate into a variety of cell lineages of all three embryonic germ layers in vitro. The hanging drop culture of ES cell suspension in the absence of leukemia inhibitory factor induces aggregation and differentiation of the cells into simple or cystic embryoid bodies (EBs). After 6 d of hanging drop culture, the resulting EBs are plated onto plastic dishes for the outgrowth culture. At d 21 after outgrowth culture, cell populations of EBs can give rise to three-dimensional gut-like structures that exhibit spontaneous contraction and highly coordinated peristalsis. The gut-like structures have large lumens surrounded by three layers: epithelium, lamina propria, and muscularis. Ganglia are scattered along the periphery, and interstitial cells of Cajal are distributed among the smooth muscle cells. The fundamental process of formation of the in vitro organized gut-like structures is similar to embryonic gastrointestinal development in vivo. The EBs at the 6-d egg-cylinder stage may have the potential to regulate developmental programs associated with cell lineage commitment and provide an appropriate microenvironment to differentiate ES cells into enteric derivatives of all three embryonic germ layers and reproduce the gut organization process in vitro.

Key Words: Embryonic stem (ES) cells; embryoid body (EB); enteric neurons; epithelial cells; gut; hanging drop culture; interstitial cells of Cajal (ICC); organogenesis; outgrowth culture; peristalsis-like contraction; smooth muscle cells.

1. Introduction

The gastrointestinal (GI) tract manifests mechanical activity such as spontaneous contraction and peristalsis and is morphologically organized by the enteric components of all three embryonic germ layers: epithelial cells (endoderm), smooth muscle and interstitial cells of Cajal (ICC) (mesoderm), and enteric neurons (ectoderm). The ICC network in the musculature of the GI tract greatly contributes to the generation of electrical pacemaker activity for GI motility *(1–5)*. This pacemaker activity manifests itself as rhythmic slow waves in membrane potential and controls the frequency and

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propagation characteristics of GI motility. Enteric neurons also innervate smooth muscle and are essential for peristalsis in GI motility *(6–9)*. Studies have reported that loss or defects in ICC or enteric neurons could be related to pathophysiology in a variety of GI motility disorders *(8–12)*. Thus, there is great interest in understanding the mechanisms that govern the differentiation of ICC and enteric neurons, not only to understand GI function but also to elucidate motility disorders. Previous in vitro analyses of gut development relied heavily on primary cultures of cells or dissociated tissues from GI sources; however, it was difficult to maintain motility function for a long period of time and to reproduce the gut organization process.

Embryonic stem (ES) cells have been used to generate particular types of cell lineages in vitro because they have pluripotency and give rise to many cell types and tissues, including representatives of all three germ layers in the embryo. ES cells are clonal cell lines derived from the inner cell mass of developing blastocysts *(13,14)*. When cultured in the presence of leukemia inhibitory factor (LIF), ES cells proliferate and remain pluripotent indefinitely. When ES cells are allowed to differentiate in a suspension culture without LIF, they form spherical multicellular aggregates, embryoid bodies (EBs) *(15–17)*. Using an EB culture system, some success has been achieved in inducing mouse ES cells to differentiate into particular types of cells, such as epidermal cells *(17)*, hematopoietic cells *(18,19)*, cardiomyocytes *(20)*, smooth muscle cells *(21,22)*, and neurons *(23–26)*. Although developmental programs associated with cell lineage commitment in EBs show remarkable similarities to those found in normal embryos *(15,16)*, the in vitro differentiation of ES cells into particular types of "organs" has not yet been shown except for a report about pancreatic islet-like organization from ES cells *(27)*.

We have previously reported that mouse ES cells formed contracting gut-like organs from EBs *(28)*. The gut-like structures had large lumens surrounded by three layers (i.e., epithelium, lamina propria, and muscularis). Ganglia were scattered along the periphery, and ICC were distributed among the smooth muscle cells. The fundamental process of formation of the in vitro-organized gut-like structures was similar to embryonic GI development in vivo *(28,29)*. These gut-like units exhibited mechanical activity, such as spontaneous contraction and highly coordinated peristalsis. Notably, the ES cell-derived gut produced electrical activity characteristic of the GI tract. The gut-like units with rhythmic contractions displayed electrical slow waves at a regular rhythm, and the developed gut-like units with highly coordinated peristalsis showed regular slow waves and spontaneous spike action potentials *(28,30)*. These results indicated that ES cells can differentiate into a functional gut-like organ in vitro that exhibits physiological and morphological properties characteristic of the GI tract. This model appears to generate the major neuromuscular cell subtypes necessary for integrated GI function and to have potential for elucidating and treating a variety of GI motility disorders.

2. Materials

2.1. Tissue Culture

1. 1X phosphate-buffered saline (PBS): prepare from 10X PBS, then aliquot into 100-mL bottles, and autoclave. To prepare 10X PBS (1 L), combine the following: 11.5 g sodium phosphate dibasic (Na_2HPO_4), 2 g potassium phosphate monobasic (KH_2PO_4), 80 g sodium

chloride (NaCl), and 2 g potassium chloride (KCl) in distilled water. It is important to dissolve the first two ingredients before adding the last two. Make the volume to 1 L. To make 1 L 1X PBS, mix 100 mL 10X PBS and 900 mL distilled water (dH_2O). Store at 4°C.
2. 500 mL Dulbecco's modified Eagle's medium (DMEM) (1X; Sigma, St. Louis, MO; cat. no. D6421) (*see* **Note 1**). Store at 4°C.
3. 500 mL fetal bovine serum (FBS) characterized and screened for ES cell growth: ES cell-quantified FBS (Invitrogen, Carlsbad, CA; cat. no. 16141-079) (*see* **Note 2**).
4. 100 m*M* mercaptoethanol 2-(ME) solution (1000X; Sigma, cat. no. M3148) (*see* **Note 3**).
5. 100 mL 10 m*M* minimum essential medium nonessential amino acids (NEAA) solution (100X; Invitrogen, cat. no. 11140-050). Store at 4°C.
6. 100 m*M* minimum essential medium sodium pyruvate solution (100X; Sigma, cat. no. S8636). Store at 4°C.
7. 100 mL penicillin-streptomycin (100X; Invitrogen, cat. no. 15140-122). Store at –20°C.
8. 1000 U/mL LIF (ESGRO, 10^7 U; Chemicon International Inc., Temecula, CA; cat. no. ESG1107).
9. 50 mg blasticidin S (Invitrogen, cat. no. R210-01).
10. 500 mL trypsin-ethylenediaminetetraacetic acid (EDTA): 0.25% trypsin and 1 m*M* EDTA-4Na (1X; Invitrogen, cat. no. 15050-065). Store at –20°C.
11. Gelatin solution: 0.1% gelatin in PBS. Sterilize by autoclave and store at 4°C (*see* **Note 4**).
12. Polystyrene 100-mm tissue culture dishes (BD Biosciences, Billerica, MA; cat. no. 353003).

2.1.1. Media

1. Medium for ES cells: ES cells are maintained on gelatin-coated 100-mm tissue culture dishes without feeder cells in supplemented DMEM in the presence of 1000 U/mL LIF. DMEM is supplemented with 10% heat-inactivated FBS, 1% 2-ME, 1% NEAA, 1% sodium pyruvate, and 0.5% penicillin-streptomycin. This media is called 10% DMEM. To prepare 1 L 10% DMEM, combine 100 mL heat-inactivated FBS, 10 mL 2-ME solution, 10 mL NEAA, 10 mL sodium pyruvate, 5 mL penicillin-streptomycin, and 865 mL DMEM.
2. Medium for EBs: EBs are maintained in hanging drops by the method described in **Subheading 3.1.4.1.** The medium for induction and maintenance of EBs is 10% DMEM in the absence of LIF.
3. Freezing medium: to prepare 10 mL freezing medium, add 1 mL dimethyl sulfoxide to 9 mL appropriate medium.

2.1.2. General Comments and Required Equipment for Tissue Culturing

As a general rule, all tissue culture protocols must be performed using sterile techniques with great attention given to using clean and detergent-free glassware. All media and solutions must be warmed to 37°C before use.

The tissue culture facility for ES cell culturing requires the following:

1. 37°C water bath.
2. Coulter cell counter Z2 series.
3. Glass pipets designated for tissue culture only (10 and 25 mL).
4. Humidified incubator at 37°C and 5% CO_2.
5. Inverted microscope with a range of phase contrast objectives (×10 to ×25) equipped with photographic capabilities.
6. Laminar flow cabinet.
7. Liquid nitrogen storage tank.
8. Pipetmen (2, 10, 20, 100, 200, and 1000 µL) designated for tissue culture use only.

9. Refrigerator (4°C) and freezer (–20°C).
10. Centrifuge.

2.2. Immunofluorescence Study

1. α-Smooth muscle actin: mouse monoclonal antibody (Sigma, cat. no. A2547).
2. Smooth muscle myosin: rabbit polyclonal antibody (Biomedical Technology Inc., Stoughton, MA; cat. no. BT-562).
3. PGP9.5: rabbit polyclonal antibody (UltraClone Limited, Isle of Wight, UK; cat. no. RA-95101).
4. c-Kit (ACK2): rat monoclonal antibody (Chemicon, cat. no. CBL-1360).
5. Alexa 594 protein labeling kit (Molecular Probes, Eugene, OR; cat. no. A-10239).
6. A confocal laser scanning microscope (Bio-Rad MRC-1024; Bio-Rad; Hercules, CA).

2.3. Electron Microscopic Study

1. 2.5% glutaraldehyde (TAAB, Berkshire, UK; cat. no. G004).
2. $CaCl_2$ (Wako, Osaka, Japan; cat. no. 039-00475).
3. Sucrose (Wako, cat. no. 196-00015).
4. Sodium cacodylate (TAAB, cat. no. S008).
5. Osmium tetroxide (OsO_4) (Merck, Darmstadt, Germany; cat. no. R3500).
6. Uranyl acetate (TAAB, cat. no. U008).
7. Ethanol (Wako, cat. no. 050-00446).
8. Propyrene oxide (Wako, cat. no. 165-05026).
9. Epoxy resin (Epon 812) (TAAB, cat. no. R3245).
10. Lead citrate (TAAB, cat. no. R3834).
11. An electron microscope (Hitachi H-7100, Hitachi, Tokyo, Japan).

3. Methods

3.1. Tissue Culture

3.1.1. Freezing Cells

Generally, freeze cells slowly and thaw them quickly. For long-term storage (indefinitely), cells must be kept under liquid nitrogen.

1. Trypsinize cells in the exponential phase of growth (~3 d in culture) from a 100-mm dish.
2. Pellet cells by centrifugation (700*g*, 5 min), resuspend in an appropriate amount of freezing medium, and gently resuspend.
3. Aliquot 1 mL cell suspension into freezing vials.
4. Immediately transfer the vials to a precooled (–20°C) StrataCooler and store it in a –80°C freezer for 24 h.
5. Transfer the tubes to liquid nitrogen.

3.1.2. Thawing Cells

When thawing vials of cells from the liquid nitrogen, one must work quickly and efficiently to maintain the integrity of the cells.

1. Immediately after the cells are thawed, transfer the entire contents of the cryovial into 10 mL medium in a 15-mL Falcon tube.
2. Collect the cells by centrifuging at 700*g* for 5 min.
3. Remove the medium and gently resuspend the cells with fresh growth medium.

4. Transfer to a tissue culture dish.
5. Allow the cells to adhere overnight in a 37°C incubator and change the medium the next day.

3.1.3. ES Cells in Culture

3.1.3.1. ES Cells

ES cells can be cultured in an undifferentiated state on feeder layers. The recombinant LIF replaces the need for maintaining ES cells on layers of feeder cells and is essential to keep ES cells from spontaneously differentiating. To form complete EBs composed of only ES cells, it is desirable to minimize the number of contaminating fibroblasts from the feeder layers. In addition, the elimination of feeder cells contributes greatly to the increased level of differentiation we see after removal of LIF in our EB culture system. Even if cultured in the presence of LIF, however, some of the ES cells are heterogeneous regarding differentiation ability under long-term repeated subculture.

To induce ES cells into gut differentiation in a highly reproducible fashion, it is important to select homogeneous ES cells that are highly undifferentiated. The ES cell line EB3 is the most confidently recommended in terms of maintaining high differentiation ability, although gut differentiation can be induced using other ES cell lines. EB3 ES cells carry the blasticidin S-resistant selection marker gene driven by the Oct3/4 promoter (active under undifferentiated status) and are maintained in media containing blasticidin S to eliminate differentiated cells ***(31)***. EB3 is a subline derived from E14tg2a ES cells ***(32)*** and is generated by a targeted integration of Oct3/4-IRES-BSD-pA vector into the Oct3/4 allele ***(31)***.

3.1.3.2. Blasticidin S Treatment

To prepare homogeneous ES cells composed of undifferentiated cells with high differentiation ability, dissociated EB3 ES cells are treated in media containing blasticidin S at the first subculture after thawing ***(31,32)***.

1. Dissociated ES cells are prepared according to **Subheading 3.1.3.3., step 10**.
2. Add 10 µg/mL blasticidin S to a 100-mm ES dish containing 10 mL medium.
3. Incubate at 37°C overnight (*see* **Note 5**).
4. Rinse the dishes three times with 10 mL 1X PBS each wash, then replace the medium with 10% DMEM and LIF.

3.1.3.3. Maintenance of ES Cells

To subculture ES cells:

1. Gently remove medium and rinse with 1X PBS.
2. Add exactly 2 mL 0.25% trypsin-EDTA to each 100-mm dish.
3. Return plate to incubator for 2–3 min until the cells float with gentle agitation.
4. When the ES cells are floating, gently pipet up and down 30 times with a 1-mL Pipetman to make a single-cell suspension.
5. For three 100-mm dishes, add 10 mL 10% DMEM to rinse each dish and collect all 36 mL into a 50-mL Falcon tube.
6. Centrifuge at 700*g* for 5 min to pellet the cells. While spinning, prepare six 100-mm dishes by labeling the cell type and passage number and add 9 mL 10% DMEM with LIF to each plate.

7. After spinning is complete, suction off the media with a Pasteur pipet; be cautious not to disturb the pellet.
8. Gently resuspend the pellet in 10 mL 10% DMEM by carefully pipeting up and down 10 times.
9. Dilute the ES cell suspension to 3.3×10^5/mL by 10% DMEM with LIF and plate 1 mL of suspension to each of the six 100-mm dishes (3.3×10^4/mL).
10. Gently agitate the plate back and forth and side to side to evenly distribute the cells and incubate at 37°C.
11. Change the ES cell medium every 2 d, and split the cultures every 3–4 d (*see* **Note 6**).

3.1.4. Differentiation of ES Cells Into the Gut

3.1.4.1. EB Formation

ES cells are derived from the inner cell mass of 3.5-d-old mouse blastocysts ***(13,14)***. When cultured in the presence of LIF, ES cells proliferate and remain pluripotent indefinitely. The hanging drop culture of ES cell suspension in the absence of LIF induces aggregation and differentiation of the cells into simple or cystic EBs ***(15–17,33,34)***. On d 2–3 of hanging drop culture, simple EBs consist of ES cells surrounded by a layer of endodermal cells. On d 3–5, cystic EBs consist of inner ectoderm-like cell layer and outer endodermal cell layer. On d 6, cystic EBs develop an additional layer of columnar ectodermal cells around a fluid-filled cavity, morphologically similar to embryos at the 6- to 8-d egg cylinder stage. At this EB stage, mesoderm-like cell populations (i.e., cardiac muscle-like beating cells) emerge from outside the endodermal cell layer. The lineage commitment and differentiation process within an EB environment reflect the development of mouse embryos (*see* **Fig. 1**).

To induce EB formation (hanging-drop culture) (*see* **Fig. 1**):

1. Dissociated ES cells are prepared as in **Subheading 3.1.3.3., step 8** (*see* **Note 7**).
2. Dilute the ES cell suspension to 3.3×10^4/mL by 10% DMEM without LIF.
3. Drop 15 µL ES cell suspension on the inside of the cover of a 100-mm tissue culture dish and make a hemisphere by surface tension. The cell density of one drop is 500 ES cells per 15 µL medium (*see* **Note 8**).
4. Turn the cover over and hang the drop of ES cell suspension (*see* **Note 9**). The medium for EB formation is not changed for 6 d.

3.1.4.2. Differentiating EB Formation

After hanging drop culture, the resulting EBs are plated onto plastic 100-mm gelatin-coated dishes. The EBs attach and begin to spread on the plate as the outgrowth culture. Within the adhesive outgrowth of EBs that are cultured in hanging drop for 2–5 d (*see* **Fig. 1A**,**B**), a cell population in EBs differentiates into sheet-like enteric smooth muscles ***(16,21,22)***. In contrast, after 6 d in a hanging drop culture (*see* **Fig. 1C**), the resulting EBs can give rise to three-dimensional gut-like structures that consist not only of smooth muscle cells but also of ICC, enteric neurons, and epithelial cells. The amount of time for EB formation in a hanging drop culture plays an important role in the capacity and pattern of EB differentiation in an outgrowth culture. When cultured in hanging drops for 6 d, ES cells form EBs with egg cylinder-like structures that consist of two cell layers—an outer primitive endodermal layer and an inner primitive

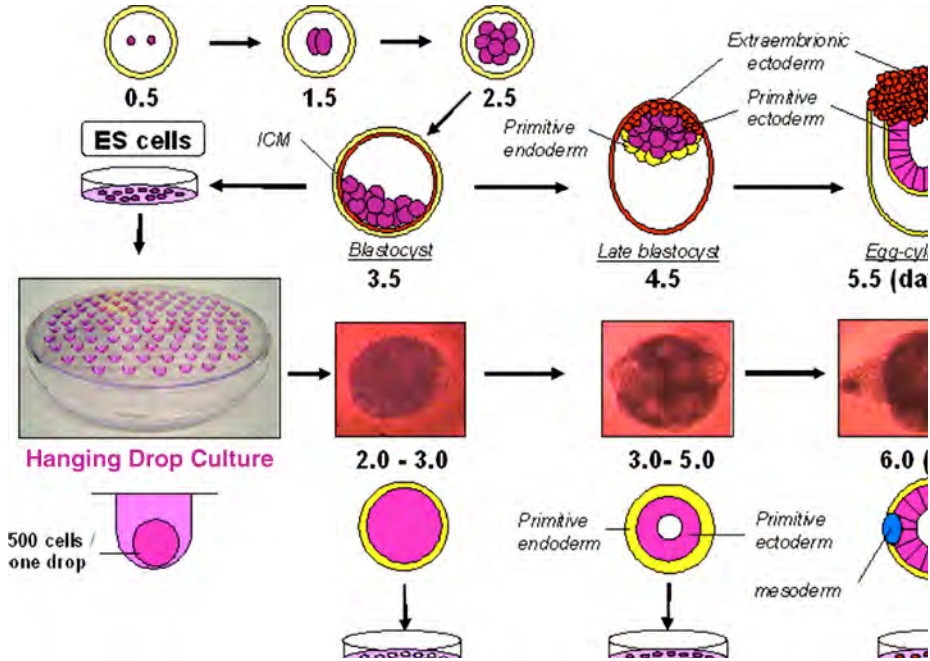

Fig. 1. Embryoid body (EB) formation by hanging drop culture and EB differentiation by outgrowth culture. The lineage commitment and differentiation process within an EB environment reflect the development of mouse embryos. (**A**) and (**B**) Within the adhesive outgrowth of EBs that are cultured in hanging drop for 2–5 d, a cell population in EBs differentiates into sheet-like enteric smooth muscles. (**C**) After 6 d in a hanging drop culture, the resulting EBs can give rise to three-dimensional gut-like structures that consist not only of smooth muscle cells but also of ICC, enteric neurons, and epithelial cells. (Please *see* the companion CD for the color version of this figure.)

ectodermal layer *(15–17,33,34)*. In a developing embryo, primitive endoderm cells form the visceral yolk sac endoderm, whereas the primitive ectoderm cells are capable of forming all the fetal tissues, including the embryonic endoderm, mesoderm, and ectoderm *(34)*. The EBs at the 6-d-old egg cylinder stage may have the potential to regulate developmental programs associated with cell lineage commitment and provide an appropriate microenvironment to differentiate ES cells into enteric derivatives of all three embryonic germ layers and reproduce the gut organization process in vitro.

The EBs cultured for 6 d in a hanging drop culture system were attached to gelatin-coated dishes, after which various types of cells, including cardiac beating muscle cells, emerged from the outgrowths.

On d 5–7 after outgrowth culture, multiple clusters within each developing EB, different from the cardiac beating muscle cells, begin to contract spontaneously with an irregular rhythm. Each contracting cluster undergoes a dramatic transformation into a hemispherical dome-like structure, with a cavity that contains fluid and solids.

On d 14, these hemispherical clusters proliferate to form more prominent three-dimensional structures with lumens and begin rhythmic contractions (*see* **Fig. 2A**).

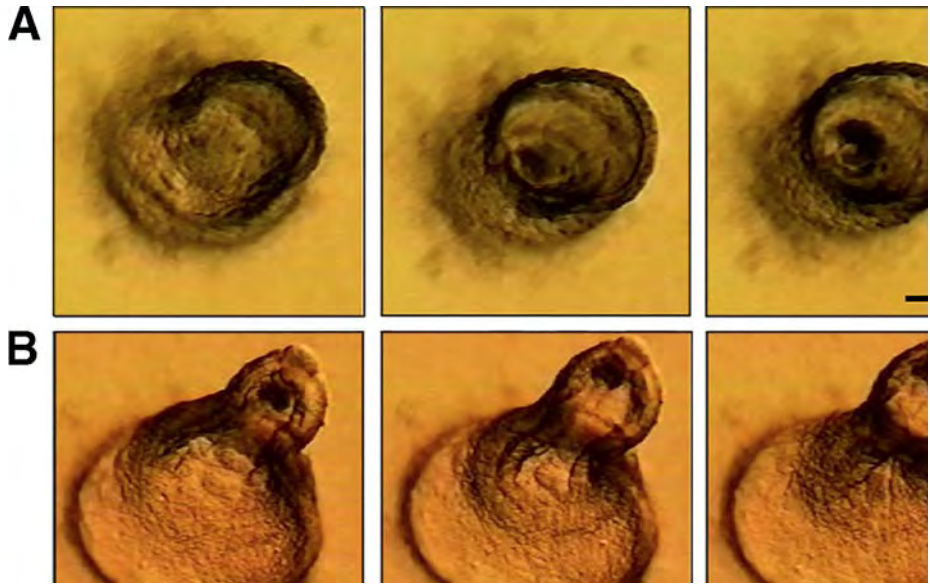

Fig. 2. Serial mechanical activities of a contracting cluster on (**A**) d 14 and (**B**) d 21 of embryoid body outgrowth culture. (**A**) The hemispherical clusters proliferated to form more prominent three-dimensional structures with lumens and began rhythmic contractions. (**B**) The hemispherical clusters showed distinct patterns of highly coordinated peristalsis-like contraction with regular rhythms. This mechanical activity was composed of periodic contraction and relaxation and was accompanied by transportation of contents. Scale bar is 200 µm. (Please *see* the companion CD for the color version of this figure.)

On d 21, the clusters show distinct and highly coordinated contraction patterns with regular rhythms. This mechanical activity is composed of periodic contraction and relaxation and is accompanied by a transportation of contents (*see* **Fig. 2B**), very similar to GI motility (i.e., peristalsis).

To differentiate EBs (outgrowth culture) (*see* **Fig. 1**):

1. Turn the cover over and wash the hemispherical drops containing EBs with 10% DMEM.
2. Collect the EBs with medium.
3. Plate 10 of the EBs to each of the 100-mm gelatin-coated dishes.
4. Gently agitate the plate back and forth and side to side to evenly distribute the EBs and incubate at 37°C.

3.2. Analysis of Differentiated Gut-Like Structures

3.2.1. Physiological Analysis

3.2.1.1. Extracellular Recording Technique

The electrical activity of the contracting clusters was examined by an extracellular recording technique. Electrical activity was recorded using a glass electrode filled with culture solution (10% DMEM) into which a platinum wire (200 µm) was inserted and with a dual-channel bioelectric amplifier (MEG-2100) under filtration at LO CUT

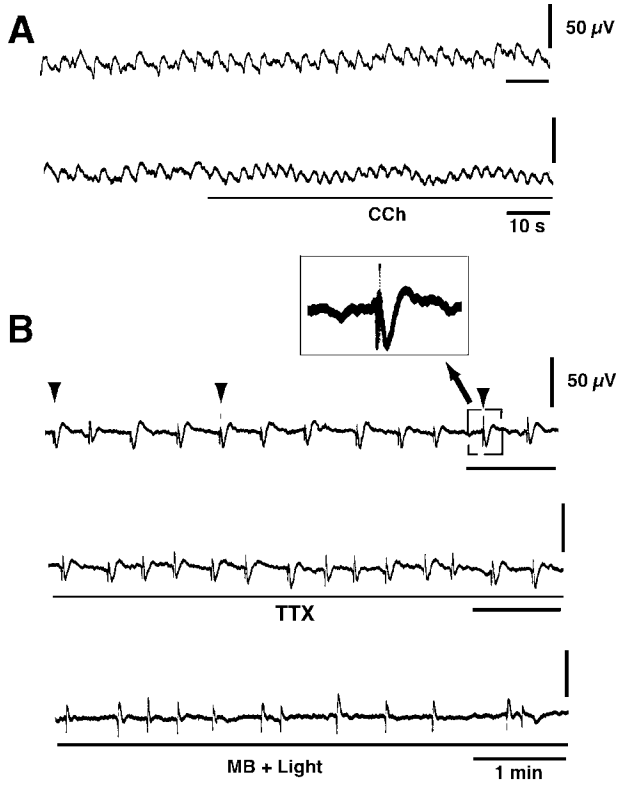

Fig. 3. Electrical activity of contracting clusters at various differentiation stages. (**A**) Slow depolarizing waves generated in rhythmic contracting clusters on d 14. Slow waves were generated at a constant frequency (13.5 cycles/min). Carbamylcholine (CCh; 1–10 μ*M*) increased the frequency of the slow waves (23.1 cycles/min); however, an L-type calcium channel blocker, nifedipine (1–10 μ*M*), did not affect the frequency of the slow waves. (**B**) Spontaneous spike action potentials (arrowheads) and regular slow waves generated in the clusters demonstrating highly coordinated peristalsis-like contractions on d 21. A neuron blocker, TTX (3.1–31 μ*M*), did not have any influence on the slow-wave component, although it abolished the spike action potentials. Subsequent to the application of TTX, MB (100 μ*M*) with light completely abolished the slow-wave component and worsened the regularity of the remaining electrical activity. These activities were recorded at 35°C. (Please *see* the companion CD for the color version of this figure.)

(0.08 Hz) and HI CUT (10 K). The inside diameter of the electrode was 0.5 mm, and its length was 2 cm. This electrode was gently placed on the surface of the clusters, to avoid injury, under an inverted microscope ***(28)***.

3.2.1.2. Electrical Activity

At d 14, clusters with rhythmic contractions displayed spontaneous slow depolarizing waves at a regular rhythm at 35°C (*see* **Fig. 3A**). The frequency was strongly dependent on the temperature and decreased at 20°C. A cholinergic agent, carbamylcholine (1–10 μ*M*),

increased the frequency of the slow waves, although an L-type calcium channel blocker nifedipine (1–10 μ*M*) did not affect the frequency of the slow waves. The slow-wave component or pacemaker activity of ICC has been proven to be insensitive to L-type calcium channel blockers *(5)*. Thus, these results suggest that clusters with rhythmic contractions consisted not only of smooth muscle cells but also of ICC networks, which generate electrical pacemaker activity for the musculature.

At d 21, as for the clusters demonstrating highly coordinated peristalsis-like contractions, we found spontaneous spike action potentials and regular slow waves, both of which were synchronized with each contraction (*see* **Fig. 3B**). A neuron blocker, tetrodotoxin (TTX) (3.1–31 μ*M*), did not have any influence on the slow-wave component; however, it abolished the spike action potentials, suggesting that these spike action potentials were elicited by neurons. Subsequent to the application of TTX, methylene blue (100 μ*M*) with light, previously shown to injure ICC *(35)*, completely abolished the slow-wave component and worsened the regularity of the remaining electrical activity. These data provided physiological evidence that the clusters with peristalsis-like contractions contained not only enteric smooth muscle cells but also ICC and enteric neurons.

3.2.2. Immunohistochemical Analysis

3.2.2.1. Immunofluorescence Stain

1. Fix tissues with Zamboni's solution at room temperature for 4 h.
2. Incubate at 4°C overnight with antibodies against α-smooth muscle actin (1:150), smooth muscle myosin (1:100), and PGP9.5 (1:5000).
3. For c-Kit immunohistochemistry, incubate tissues with Alexa 594-conjugated ACK2 (1:100) for 10 min.
4. Fix with a 4% paraformaldehyde solution for 30 min *(4)*.
5. Examine localization of fluorescence with a confocal laser scanning microscope *(28,29)*.

3.2.2.2. Immunohistochemical Study

On d 14, immunoreactivity for both smooth muscle-specific actin and myosin was observed in the wall surrounding the lumen and spreading out from the clusters (*see* **Fig. 4A**,**B**). Immunoreactivity for c-Kit, a useful marker for ICC *(1,2,36,37)*, was detected in clusters that had spontaneous contractions at a regular rhythm and generated electrical slow waves. The c-Kit immunopositive (c-Kit+) cells were mostly multipolar.

On d 21, the c-Kit+ cells formed a distinct and dense network (*see* **Fig. 4C**). This network structure of c-Kit+ cells is similar to that of ICC at the level of the mesenteric plexus in a murine embryo or neonate *(4)*. Neuronal marker PGP9.5 immunoreactivity was observed in clusters that showed peristalsis-like contractions (*see* **Fig. 4E**). PGP9.5 immunopositive (PGP9.5+) cells were also distributed outside the clusters, where they formed ganglion-like structures. PGP9.5+ fibers from ganglions located close to the clusters were often seen projecting into the contracting clusters (*see* **Fig. 4D**). PGP9.5+ cells in the cluster were distributed in a solitary manner and seldom formed a ganglion (*see* **Fig. 4E**). Most of them were unipolar, with a protruding single axon-like process with varicosities, although a few were multipolar with prominent dendrites (*see* **Fig. 4F**).

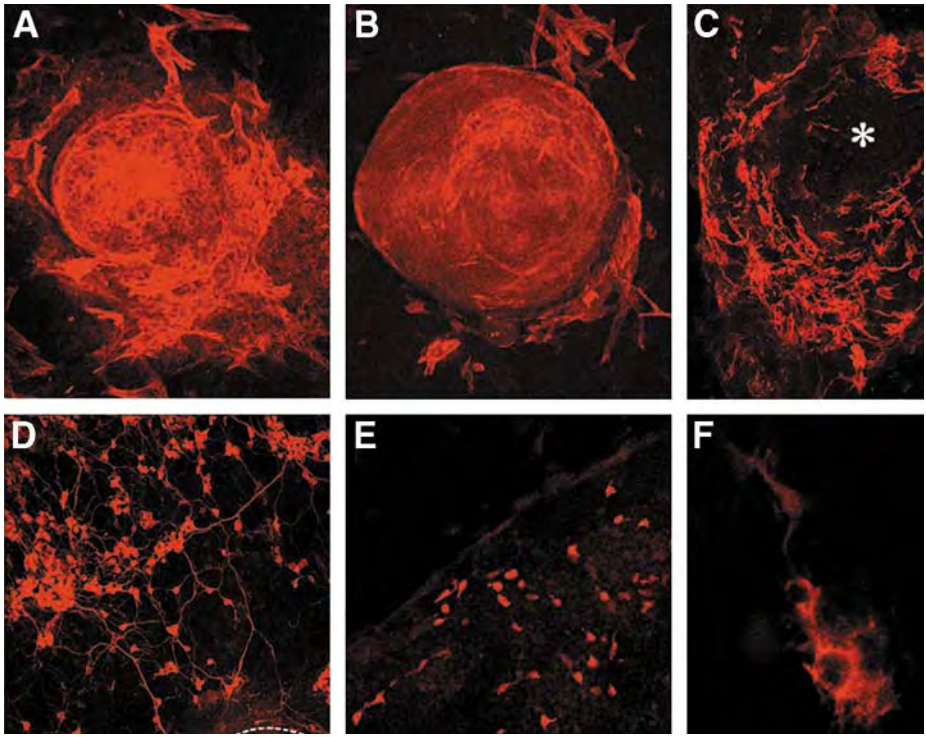

Fig. 4. Immunohistochemistry for smooth muscles, interstitial cells of Cajal (ICC), and neurons in contracting clusters on d 14 and 21 of EB culture. (**A**) A hemispherical dome-like cyst showing smooth muscle actin immunoreactivity and some immunopositive cells spreading out from the cluster. (**B**) Immunoreactivity for smooth muscle myosin was detected on a dome-like contracting cluster and some surrounding cells. (**C**) A large number of c-Kit-positive cells were identified in the wall of the dome-like structure surrounding the lumen (*) and formed a distinct and dense network. The c-Kit-positive cells were mainly multipolar and were similar to ICC at the level of the myenteric plexus of the mouse gastrointestinal tract; however, they did not form a single layer but were scattered throughout the muscle layer. (**D**) PGP9.5+ cells outside the cluster showing ganglion-like structures and projecting their fibers into a contracting cluster (broken line). (**E**) PGP9.5+ cells located in the wall of a contracting cluster. They did not form any ganglions in the cluster. (**F**) A small number of PGP9.5+ cells showing a typical configuration of the enteric neuron with several dendrites. Scale bar represents the following sizes: (**A–C**) 200 µm; (**D**) 300 µm; (**E**) 150 µm; and (**F**) 20 µm. (Please *see* the companion CD for the color version of this figure.)

3.2.3. Electron Microscopic Analysis

3.2.3.1. Fixation and Staining

1. Fix tissues with 2.5% glutaraldehyde, 1.25 m*M* $CaCl_2$, and 3% sucrose in a 0.05 *M* cacodylate buffer (pH 7.4) at room temperature for 3–4 h.
2. Wash tissues three times for 10 min with 6% sucrose in a 0.05 *M* cacodylate buffer (pH 7.4) at room temperature for 3–4 h.

3. Postfix with 1% osmium tetroxide (OsO_4) and 6% sucrose in a 0.05 *M* cacodylate buffer (pH 7.4) for 1 h.
4. Stain *en bloc* with uranyl acetate for 1 h.
5. Dehydrate with 70, 80, 90, and 95% ethanol for 10 min; 100% ethanol three times for 15 min; and propyrene oxide for 30 min.
6. Embed in epoxy resin at 60°C for 2 d.
7. Double stain ultrathin sections with 3% uranyl acetate for 10 min and 3% lead citrate for 5 min.
8. Examine with an electron microscope ***(28,29)***.

3.2.3.2. Ultrastructural Characteristics of the Contracting Clusters

Electron microscopic analysis confirmed the cellular components of the cluster. The wall had three distinct layers in a semithin section stained with toluidine blue (*see* **Fig. 5A**). The innermost was a flat and single layer of epithelium, which did not form any plica, intestinal villus, or crypt. Most of the epithelial cells were columnar type, with fewer and shorter microvilli than those in epithelia in the mouse GI tract. Well-developed goblet cells were common, and tuft cells were also scattered among the columnar cells (*see* **Fig. 4B,C**). We considered it remarkable that the enteroendocrine cells that contained numerous secretory granules were well differentiated (*see* **Fig. 4D**). All of these epithelial cells were separated from the connective tissue by a basal lamina (*see* **Fig. 4A**).

The outermost layer was composed of prominent smooth muscle cells filled with filament-like structures (i.e., thick, thin, and intermediate filaments) in their cytoplasm and had membrane-free or -bound dense bodies (*see* **Fig. 4E**). These smooth muscle cells ran parallel to one another and formed fascicles or sheets corresponding to circular or longitudinal muscle layers, respectively, although the muscularis mucosa could not be identified. We confirmed the occurrence of ICC in the muscle layer by ultrastructural features such as electron-dense cytoplasm, numerous mitochondria, and caveolae on the cell membrane (*see* **Fig. 4F**).

ICC were connected to each other by their long cell processes surrounding smooth muscles, suggesting a multipolar cell type. Nerve fibers were also seen in the muscle layer, although such enteric ganglia as Auerbach's or Meissner's plexuses were seldom observed in the cluster. The third layer, between the epithelium and muscle layer, was a thin connective tissue corresponding to the lamina propria or submucosa (*see* **Fig. 4A**). There were many fibroblasts and collagen fibers present; however, the absence of both blood and lymphatic vessels was striking. The outer surface of the cluster was lined with a flat, thin monolayer, similar to the serosa.

4. Notes

1. This medium is high in glucose and contains 4500 mg/L D-glucose and L-glutamine, pyridoxine, HCl, and $NaHCO_3$.
2. Serum is heat inactivated in the following manner: thaw the bottle of serum overnight at 4°C, warm in a 55°C water bath with constant mixing, then continue to incubate serum for 30 min, agitating every 10 min. The serum is then aliquoted into sterile 100-mL bottles in the tissue culture hood and allowed to cool before tightening the bottle and freezing. It is important to cool the bottles to avoid breakage. Serum may be stored at –20°C for 6–12 mo.

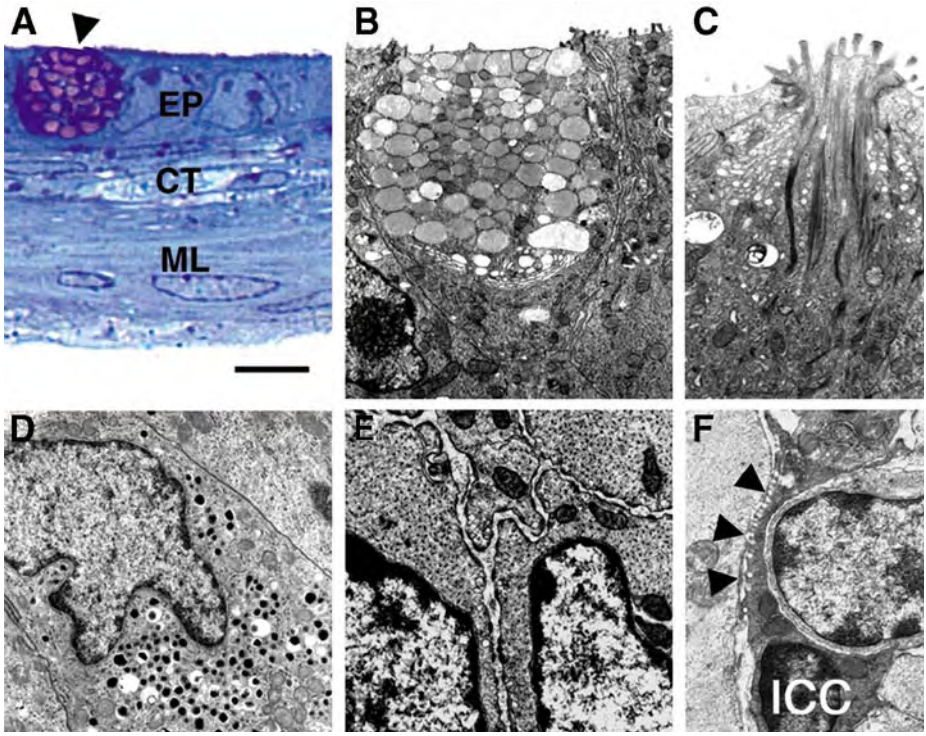

Fig. 5. Semithin section stained with toluidine blue and electron micrographs of contracting clusters on d 21 of embryoid body culture. **(A)** A cross section of the wall stained with toluidine blue clearly showing three layers: epithelium (EP), connective tissue (CT), and smooth muscle (ML). A goblet cell (arrowhead) is prominent in the EP, similar to mouse GI epithelia. **(B)** Electron microscopic image confirming many mucous globules in the apical cytoplasm of a goblet cell. **(C)** Tuft cells were less common, although observed among columnar cells in the EP and were characterized by microvilli with long bundles of straight filaments extending from the core of the microvilli and small vesicles in the apical cytoplasm. **(D)** An enteroendocrine cell containing many electron-dense secretory granules in the basal cytoplasm. **(E)** Smooth muscle cells in the muscle layer showing typical features of myofilaments, dense bodies, and caveolae. **(F)** ICC had an electron-dense cytoplasm, many caveolae (arrowheads), mitochondria, and long cell processes surrounding smooth muscle cells. Scale bar in **(A)** is 100 µm. Scale bar represents the following sizes: **(B)** 20 µm; **(C)** 15 µm; **(D)** 15 µm; **(E)** 10 µm; and **(F)** 15 µm. (Please *see* the companion CD for the color version of this figure.)

3. Prepare 100 m*M* stock solution (1000X) by adding 0.1 mL 2-ME to 14.1 mL PBS. Filtrate with 0.2-µm filter and store up to 4 wk at 4°C.
4. All dishes and plates should be gelatinized before use by the following method:
   a. Add gelatin solution.
   b. Keep 10 min at room temperature.
   c. Aspirate thoroughly.
5. Treatment with blasticidin S can allow only differentiated ES cells to float. Adhesive cells are homogeneous ES cells that consist of only undifferentiated cells.

6. It is important not to keep ES cells in culture for long periods to maintain pluripotency. Extensive culturing will result in abnormal karyotypes and inconsistent differentiation. Efficient differentiation of ES cells into gut-like structures requires uniformity of undifferentiated ES cells with a high quality of pluripotency. It is for this reason that we generally keep ES cells in culture up to the sixth passage before new cells are thawed, and we prepare ES cells treated with blasticidin S at the every first subculture.
7. Before dissociation of ES cells, rinse the dishes three times with 10 mL 1X PBS each wash to remove LIF.
8. In our pilot study, the cell density of one drop, 500 ES cells per 15 μL media, is an appropriate condition to achieve better EB differentiation. A drop over 30 μL in volume is unable to withstand gravity and surface tension. A large EB composed of over 500 ES cells is not capable of inducing the efficient differentiation into gut-like structures because of deficiency of oxygen and nutrition in medium at the center of the aggregates.
9. To avoid drying in a dish, store 10 mL 1X PBS in the bottom of each dish.

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19

Formation of Gut-Like Structures In Vitro From Mouse Embryonic Stem Cells

Shigeko Torihashi

Summary

Embryonic stem (ES) cells have the potential to differentiate into all cell types originating from the three germ layers; however, there are still few reports about the formation of functional organs from embryonic stem cells. Recently, we reported that by hanging drops of mouse ES cells, embryoid bodies (EBs) formed gut-like structures in vitro composed of three layers corresponding to the epithelium, lamina propria, and musculature. The morphological features and the process of formation are similar to gut and its organogenesis in vivo. Thus, this is a good model for development of the gut and a useful tool for analysis of the factors required for gut organogenesis. The protocol basically involves a method of hanging drops to make EBs, which are then plated on coated dishes for outgrowth. EBs develop to form gut-like structures when induced to spontaneously enter a program of differentiation in vitro without addition of any extrinsic factors.

Key Words: Development; embryoid body; endoderm; epithelium; ES cells; gut; hanging drop; interstitial cells of Cajal; lamina propria; musculature; organogenesis; pacemaker cells; smooth muscle; spontaneous contraction.

1. Introduction

Gut organogenesis involves all three germ layers and shows regional tissue specificity from the oral to the anal ends. The gut tube is also composed of three distinct layers (i.e., epithelium, lamina propria, and muscularis). They originate from endodermal epithelium and mesenchyme interacting with each other. Because of these complexities, little is known about the mechanism underlying gut organogenesis.

It has been previously reported that mouse embryonic stem (ES) cells form contracting gut-like structures *(1,2)*. Their morphological features are similar to those in the mouse gastrointestinal tract (**Figs. 1** and **2**). Gut-like structures have differentiated epithelium provided with enterocytes, goblet cells, enteroendocrine cells, and tuft cells (brush cells). On the outside of the connective tissue layer corresponding to the lamina propria, there is a musculature with interstitial cells of Cajal that act as pacemaker cells,

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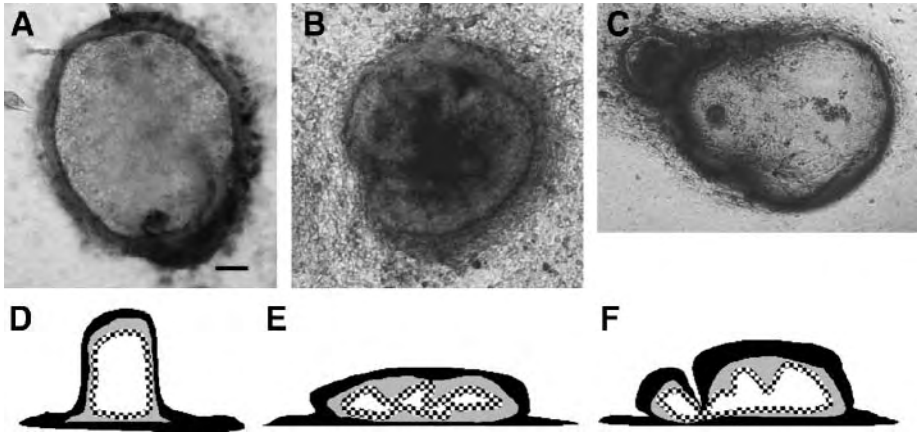

Fig. 1. Shapes of gut-like structures and schematic view of their cross-sections. (**A**) A dome-like structure with an expanded lumen. The wall of the structure is relatively thin, as indicated in **D**. (**B**) Flattened and spherical structure. There are plicae or folds of the epithelium, and its wall is thick, as shown in **E**. (**C**) Multivacuolar-type structure. The lumen is completely divided into two separate parts or sometimes connected by a narrow channel. Gut-like structures are composed of three layers, as seen in **D–F**: epithelia (dotted lines), connective tissue layers (gray zones), and the muscularis (black layers). Scale bar = 100 µm. (Reproduced from **ref.** *2* with permission of Blackwell Publishing.)

producing periodic rhythms for spontaneous contractions. A small number of enteric neurons are also present in the wall of the gut-like structures.

These fundamental features are similar to those in the neonate mouse gut except that they lack blood vessels. The physiological properties, showing spontaneous contractions, also mimic those in the mouse gastrointestinal tract *(3)*. The developmental process of gut-like structures was investigated morphologically and follows a course similar to gut organogenesis. They first form stratified squamous epithelium and then differentiate into a smooth muscle layer outside the epithelium, followed by differentiation into subtypes of epithelial cells. Morphological observations suggested that a gut-like structure at about 14 d of outgrowth culture corresponded to the guts of newborn mice. Furthermore, transcriptional factors crucial for development of endoderm and gut organogenesis are expressed in the gut-like structures in a manner similar to that in vivo (in preparation). These findings indicate that the same molecular mechanisms found in gut development in vivo are also involved in the formation of gut-like structures from ES cells. Thus, in vitro formation of gut-like structures is a good model for gut organogenesis.

The present protocol basically consists of hanging drops to make embryoid bodies (EBs) and then plating them on coated dishes for outgrowth. EBs develop to form gut-like structures when induced to spontaneously enter a program of differentiation in vitro without addition of any extrinsic factors. Of crucial importance to this system, therefore, are fetal bovine serum (FBS) and the population (cell number) of ES cells, EBs in dishes, and the number of passage times before the hanging drop process.

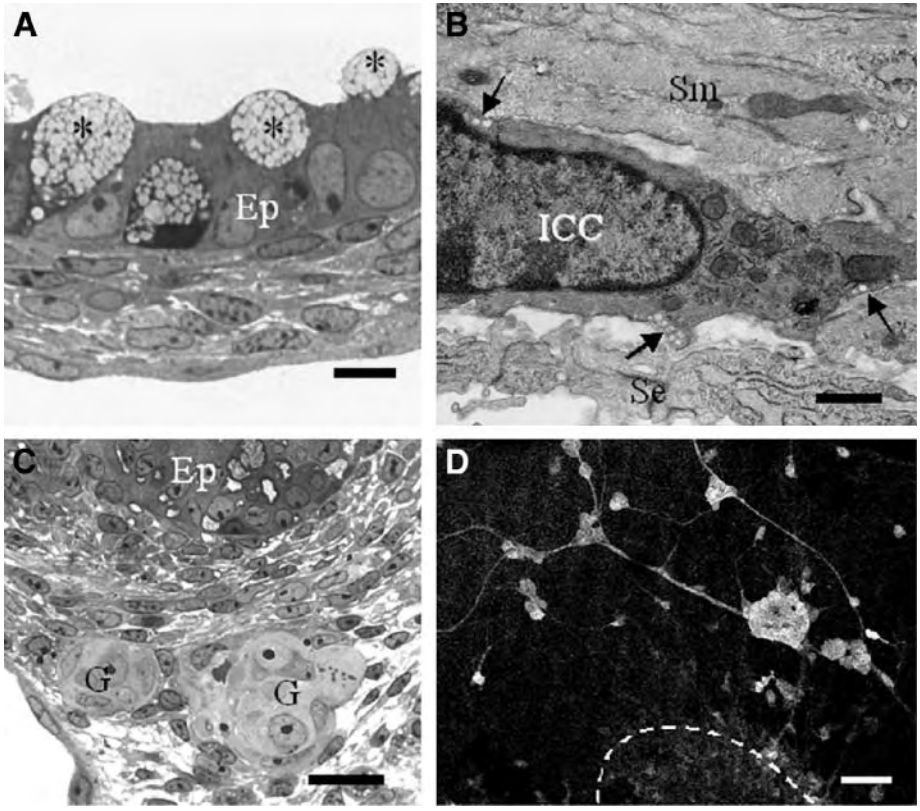

Fig. 2. Fine structures of cellular components of gut-like structures. (**A**) Epithelium (Ep) is composed of columnar epithelial cells, including goblet cells (asterisks) as seen in a semithin section stained with toluidine blue. They are surrounded by a connective tissue layer and muscularis. Scale bar = 10 μm. (**B**) The muscularis, demonstrated by electron microscopy, is packed mainly with smooth muscles (Sm) and surrounded by a serosa (Se). Sometimes interstitial cells of Cajal (ICC), characterized by an electron-dense cytoplasm, many mitochondria, and caveolae (arrows), are observed. Scale bar = 1 μm. (**C**) In toluidine blue semithin sections, a few nerve ganglia (G) are observed beneath the epithelium (Ep). Scale bar = 30 μm. (**D**) Immunohistochemistry for anti-PGP9.5 antibody in the whole-mount preparation demonstrates that many nerve elements, including ganglia, surround the gut-like structures, shown by a broken line, and that only a few of them intrude into the gut-like structures. Scale bar = 50 μm. (Reproduced from **ref.** *2* with permission of Blackwell Publishing.)

2. Materials

2.1. ES Cells (Feeder-Free ES Cells)

G4-2 is derived from EB3 (a gift from Dr. Niwa, Riken, Japan), a subline originated from E14tg2a and carrying the enhanced green fluorescent protein (EGFP) gene under the control of the CAG expression unit. It also carries the blasticidin S-resistant selection

marker gene driven by the Oct3/4 promoter and is maintained in medium containing 10 μg/mL blasticidin S to eliminate differentiated cells. G4-2 is one of the ES cells growing under the feeder-free condition. Cells should be kept in a deep freezer under –80°C or in a gas phase of a liquid nitrogen container.

2.2. Medium for ES Cells

1. Dulbecco's modified Eagle's medium (DMEM) containing 4.5 mg/mL D-glucose, L-glutamine, pyridoxine-HCl, and $NaHCO_3$ (Sigma, St. Louis, MO; cat. no. D-5796). Store at 4°C.
2. 10 m*M* nonessential amino acids (NEAA) (100X; Gibco Invitrogen Corp., Carlsbad, CA; cat. no. 11140-050). Store at 4°C.
3. 100 m*M* sodium pyruvate solution (100X; Gibco Invitrogen, cat. no. 11360-070). Store at 4°C.
4. 25 g 2-mercaptoethanol (Nacalai tesque, Kyoto, Japan; cat. no. 21438-82): dilute 70 μL/100 mL in phosphate-buffered saline (PBS). Filter and store 10-mL aliquots at –20°C.
5. Penicillin-streptomycin (10,000 U/mL) (Gibco Invitrogen, cat. no. 15140-122). Store 5-mL aliquots at –20°C.
6. 500 mL fetal bovine serum (FBS) (Vitromex, GmbH, Germany; cat. no. VM S1500, batch no. F000210802) (*see* **Note 1**).
7. PBS without calcium chloride or magnesium chloride (Gibco Invitrogen, cat. no. 21600-010). Dissolve in 1 L distilled water (dH_2O), filter or autoclave, and store at 4°C.
8. Leukemia inhibitory factor (LIF) (ESGRO 10^7 U/mL; Chemicon, Temecula, CA; cat. no. ESG1107). Store 100-μL aliquots at 4°C.
9. Trypsin-ethylenediaminetetraacetic acid (EDTA) (0.25% trypsin-1 m*M* EDTA solution) (Nacalai tesque, cat. no. 3554-64). Dilute three times with PBS and store 50-mL aliquots at 4°C.
10. Gelatin (Sigma, cat. no. G-1890): dissolve 1 g/L in PBS and store at 4°C. after autoclaving.
11. Blasticidin S hydrochloride (Funakoshi, Tokyo, Japan; cat. no. KK400): dissolve 1 mg/100 μL in distilled water, filter, and store at –20°C.

2.3. Equipment

1. 100-mm tissue culture dishes (Corning, NY; cat. no. 430167).
2. 60-mm tissue culture dishes (Falcon, Franklin Lakes, NJ; cat. no. 3004).
3. 100-mm Petri dishes.
4. 10-mL pipet (Falcon, cat. no. 7551).
5. Pipet Aid (Falcon, cat. no. 7590).
6. Pipet (10, 100, and 1000 μL) (Eppendorf, Hamburg, Germany; cat. no. 94632, 94634, and 94636, respectively).
7. Millex syringe-driven 0.45-μm filter unit (Millipore, Bedford, MA; cat. no. SLHV R25 LS).
8. Microdispenser (Bio-craft, Tokyo, Japan; model no. 502) (*see* **Note 2**).
9. Hemocytometer (Burker-Turk deep 1/10 mm) (Erma, Tokyo, Japan; cat. no. 5855).
10. 37°C water bath.
11. Humidified incubator at 5% CO_2 and 37°C.
12. Inverted microscope.
13. Centrifuge.

2.4. ES-DMEM

Mix 5 mL nonessential amino acids, 5 mL sodium pyruvate, 5 mL 2-mercaptoethanol, and 2.5 mL penicillin-streptomycin. Filter the mixture with a Millex syringe-driven filter unit and add the filtered mixture to DMEM after extraction of the same volume of

DMEM. Aliquot to 45 mL and add 5 mL FBS to each. This medium is called ES-DMEM (LIF minus ES-DMEM). Add 5 µL LIF to 50 mL ES-DMEM to make LIF+ ES-DMEM.

3. Methods

3.1. Expansion of ES Cells With LIF

1. Incubate a frozen vial of ES cells in a water bath at 37°C (*see* **Note 3**).
2. Just before cells thaw completely (vial is still cold), transfer them to a 15-mL centrifuge tube and add 10 mL ES-DMEM.
3. Collect the cells by centrifuging at 240*g* for 5 min.
4. Remove the medium and resuspend cells into 1 or 2 mL ES-DMEM.
5. Seed cells into two 100-mm tissue culture dishes coated with 0.1% gelatin and filled with 10 mL LIF+ ES-DMEM (*see* **Note 4**).
6. Incubate cells at 37°C and change the medium 2 d later.
7. At 3 d later, or when cells increase to 70% confluence, cells are processed for passage.
8. Rinse dishes twice with 5 mL warmed PBS.
9. Add 3 mL diluted trypsin-EDTA at 37°C to each dish and return dishes to the incubator at 37°C for 1 min (*see* **Note 5**).
10. Pipet up and down about 15 times using 10-mL pipet to make a single-cell suspension.
11. After confirming under the microscope that cells are completely isolated, collect cells of two dishes into a 50-mL tube filled with 40 mL PBS and stop the enzyme reaction.
12. Centrifuge at 240*g* for 5 min at 4°C to pellet the cells. While centrifuging, prepare a hemocytometer and two 100-mm tissue culture dishes coated with 0.1% gelatin. Add 10 mL LIF+ ES-DMEM to each dish and label the dishes.
13. After centrifuging and suctioning off PBS, add 1–4 mL ES-DMEM and agitate gently.
14. Count cell numbers using the hemocytometer and calculate the number of collected cells.
15. Plate 3×10^5 cells to each 100-mm dish containing 10 mL LIF+ ES-DMEM and gently agitate the dishes to distribute the cells evenly. Incubate cells at 37°C. About 2 d after they reach confluence, repeat passage to increase cells exponentially.

3.2. Hanging Drops to Make EBs (Fig. 3)

1. After two or three passages and when cells increase exponentially, process them for hanging drops. Continue sequential passages with remaining cells.
2. Collect cells by treatment with trypsin-EDTA as described in **Subheading 3.1.**, count cells using the hemocytometer, and calculate the number of collected cells.
3. For hanging drops, cells must be diluted with ES-DMEM to $2.3–4.6 \times 10^4$/mL containing 500–1000 cells per 15 µL in each drop (*see* **Note 6**).
4. Suspend cells in a 50-mL tube filled with the required amount of ES-DMEM and gently agitate up and down.
5. Distribute 3 mL PBS or distilled water to each 100-mm Petri dish to retain the humidity. Open a 100-mm Petri dish and invert the lid.
6. Using a microdispenser, deposit drops (15 µL ES-DMEM containing 500–1000 ES cells) on the inverted lid.
7. After plating 80 drops on the lid, restore the lid gently to its original position and cover the dish. Drops are now dripping from the lid of the dish.
8. Incubate dishes with the drops in an incubator at 37°C for 6 d, avoiding vibration.
9. After 6 d, confirm the formation of EBs in each drop by a dissection microscope.
10. Open the dish and gently invert the lid again.

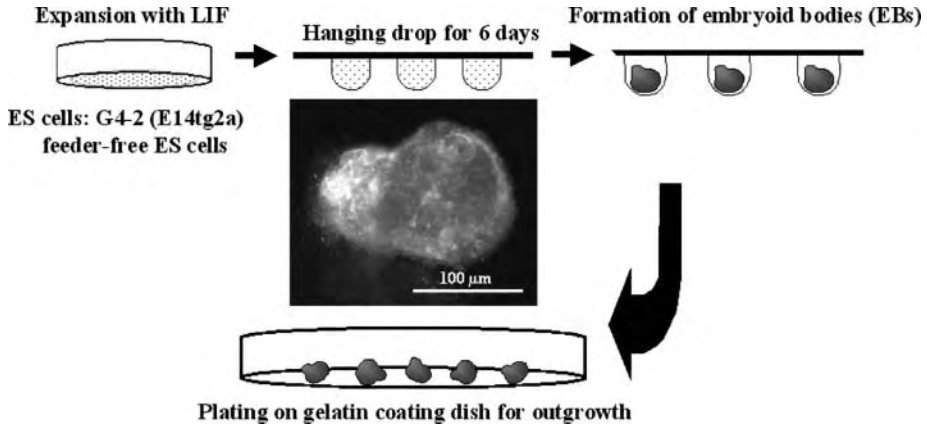

Fig. 3. Schematic view of the method. The protocol is composed of three main steps. (1) Expansion of undifferentiated embryonic stem (ES) cells in the culture with leukemia inhibitory factor (LIF) 1000 U/mL. (2) Hanging drops to make embryoid bodies (EBs) without LIF for 6 d. Each drop contains 15 μL ES-DMEM and 500–1000 ES cells. After 6 d, an EB is formed in each drop. (3) Plating EBs to gelatin-coated dishes. EBs grow and develop to form gut-like structures.

11. Tilt the lid and carefully flush down EBs with 3 mL ES-DMEM using a 10-mL pipet.
12. Gently collect EBs in the pipet and transfer them to a gelatin-coated 60-mm tissue culture dish.
13. Keep dishes containing EBs in an incubator at 37°C and avoid any disturbance. Designate the stage of EB as EB0 once EBs are transferred to the 60-mm tissue culture dish. Change medium every other day, designating as EB2, 4, 6, and so on.

3.3. Formation of Gut-Like Structures (Fig. 4)

The formation process of gut-like structures is not well regulated, and each EB follows a different time-course. The general time-course, however, is as follows:

1. EB0. The EBs assume a cylindrical ovoid shape consisting of a small head and a large body for a total length of 100–150 μm.
2. EB1. EBs attach to the dish. The contraction of cardiac muscles begins.
3. EB5. Future gut-like structures are recognizable as a cluster of mesenchymal cells with or without lumens.
4. EB10–14. Gut-like structures begin spontaneous contractions and start to disintegrate after EB28.

4. Notes

1. FBS is very important in the culture ES cells. The lot check using ES cells is necessary and a large number of bottles of high-quality FBS should be maintained. Use the same lot during each series of experiments. Heat inactivation (57°C for 30 min) should be performed before use.
2. A pipet is used to make 15-μL drops of ES-DMEM containing ES cells. A full pipet can make about 15 drops. A sequential pipeter such as the Eppendorf Multipette plus and Combitip plus may also be useful.

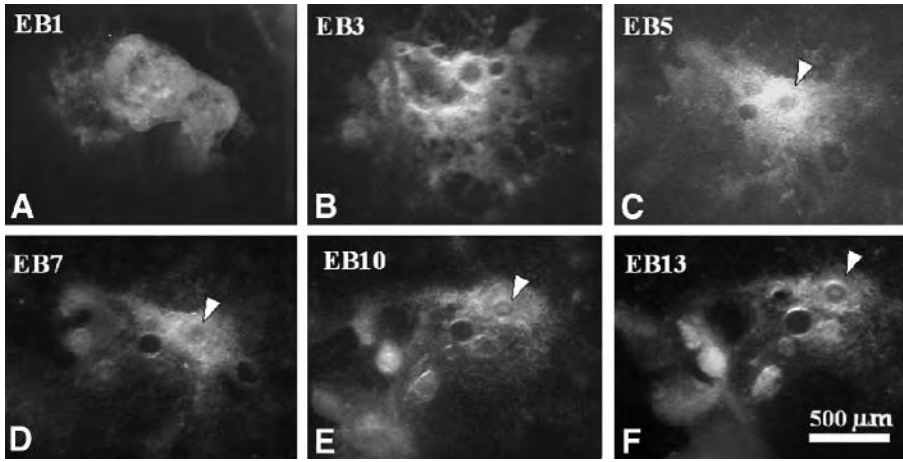

Fig. 4. Outgrowth and formation of gut-like structures from single embryoid bodies (EB) after plating to a gelatin-coated dish. **A–F** show the same EB at EB1, EB3, EB5, EB7, EB10, and EB13, respectively. Arrowheads indicate the same gut-like structure, and scale bar applies in all panels.

3. One frozen vial contains 2–3 × 10^6 cells/0.5 mL.
4. Coat 100-mm tissue culture dishes with 3 mL 0.1% gelatin in PBS at least 10 min before use. They can be stored in an incubator for 1 wk or more.
5. Do not stack dishes on one another in the incubator. Each dish should be rack mounted to maintain an even temperature.
6. The number of ES cells in a drop depends on their growth rate. If they increase more than six times during a single passage for 2 d, then 500 cells in one drop is enough. About 80 drops can be made per dish, meaning that 1.2 mL diluted cell suspension would be necessary for one dish.

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20

In Vitro Derivation and Expansion of Endothelial Cells From Embryonic Stem Cells

Kara E. McCloskey, Steven L. Stice, and Robert M. Nerem

Summary

Vascular endothelial cells or endothelial progenitor cells derived from stem cells could potentially lead to a variety of clinically relevant applications, including cell-based therapies and tissue engineering. Embryonic stem (ES) cells serve as an excellent in vitro system for studying differentiation events and for developing methods of generating various specialized cells for future regenerative therapeutic applications. Two obstacles associated with using embryonic stem cells include (1) isolating homogeneous populations of differentiated cells and (2) obtaining terminally differentiated cell populations that are capable of proliferating further. Here, we describe methods for isolating purified proliferating populations of endothelial cells from mouse ES cells using Flk-1-positive cells, vascular endothelial growth factor supplementation, and a highly selective manual selection technique. This methodology, although rigorous, overcomes two current obstacles in ES derivation and culture by generating highly purified (>96%) populations of actively proliferating endothelial cells from mouse ES cells. Using this in vitro derivation procedure, millions of cells at various stages of differentiation may be obtained and expanded up to 25 population doublings.

Key Words: Embryonic stem cells; endothelial cells; endothelial progenitor cells; Flk-1; vascular progenitor cells; VEGF.

1. Introduction

Stem cell research has gained significant interest because of the potential for these cells to repopulate specific cell tissues that may be damaged or missing owing to a pathological disorder. Stem cells are unspecialized cells, able to differentiate into a variety of specialized cell types, and exhibit the capacity for self-renewal and pluripotency. Pluripotent cells are undifferentiated stem cells that are able to give rise to *all* cell types derived from the three embryonic germ layers: mesoderm, endoderm, and ectoderm. Currently, the only known sources of pluripotent stem cells are derived from early embyros and pregonadal fetal tissue ***(1,2)***.

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Embryonic stem (ES) cells are derived from the inner cell mass of a blastocyst stage embryo. When the inner cell mass is removed from the embryo and cultured under appropriate conditions, including leukemia inhibitory factor (LIF), these embryonic-derived cells will proliferate indefinitely while maintaining the potential to form derivatives of the three germ layers *(3)*.

Vascular endothelial cells (ECs) or endothelial progenitor cells derived from stem cells could potentially lead to a variety of clinically relevant applications. Because it is well known that ECs inhibit platelet adhesion and clotting, ECs are needed for lining the lumen of a synthetic or tissue-engineered vascular graft *(4,5)*. These cells could also be used for vascularizing tissue-engineered materials prior to implantation or for therapeutic strategies to repair and revascularize ischemic tissue in patients exhibiting vascular defects by either directly injecting the cells *(6)* or implanting them in a tissue-engineered vascular patch. ECs might also be an especially attractive cell source for cardiac repair. It has been shown that ECs are able to transdifferentiate into cardiomyocytes under co-culture conditions *(7)*. Moreover, because ECs line the lumen of blood vessels and can release proteins directly into the bloodstream, they are ideal candidates to be used as vehicles for gene therapy; because ECs are the key players in angiogenesis and vasculogenesis, ECs are commonly used to study these basic biological phenomena.

Vasculogenesis is a process in which hemangioblasts, EC precursors from mesodermal origins, grow and organize to form vascular networks. Currently, little is known about the mechanisms that regulate the growth and differentiation of ECs. Some studies have been conducted using mouse ES cells to investigate cellular events in vasculogenesis *(8–11)*. One particular molecule, fetal liver kinase (Flk)-1, is expressed on vascular progenitor cells *(8)*. These early Flk-1-positive, embryonic-derived cells have the potential to differentiate into both endothelial and blood cells and have the capability of mimicking the vascularization process in vitro.

Previously, it was believed that a three-dimensional structure, called an embryoid body, was needed to partially mimic the spatial organization in the embryo. Evidence has demonstrated that it is possible to differentiate cells into Flk-1-positive-expressing cells by culturing these cells on type IV collagen-coated dishes. Further differentiation into blood cells and vascular cells required isolation of the Flk-1 cells from Flk-1-negative cells *(9)*. At this stage, vascular endothelial growth factor (VEGF) promoted EC differentiation of Flk-1-positive cells *(8,9)*. Flk-1-positive cells cultured in VEGF also formed tubelike structures in vitro, mimicking vasculogenesis *(9)*. Other molecules, such as platelet-EC adhesion molecule (PECAM)-1 and vascular endothelial cadherin (VE-cadherin) were also shown to be involved in the vascular differentiation process *(10,11)*.

Here, we present methodology that allows derivation of vascular progenitor cells and ECs from mouse ES cells (ES-D3). The complete process takes approx 1 mo. The mouse ES cells are first expanded for 1 wk without fibroblast feeder cells. The cells are allowed to differentiate on collagen type IV without LIF supplementation for 4 d and then sorted for expression of the endothelial progenitor surface marker Flk-1. The Flk-1-positive cells consist of both endothelial and smooth muscle-like vascular cells and therefore are purified further using manual selection of EC colonies. It should be noted that although

it is reasonable to expect that the protocols described will translate to most mouse ES cell lines, and more generally to human ES cell lines (although differences in antibodies and timing/kinetics for specific stages of differentiation and cell growth should be expected), the derivation procedure described here has been produced in our laboratory using mouse ES-D3 cells only.

2. Materials

2.1. Tissue Culture

1. ES-D3 cells (American Type Culture Collection [ATCC], Manassas, VA; cat. no. CRL-1934).
2. Primary mouse embryonic fibroblasts or STO cells (ATCC, cat. no. CRL-1503).
3. Dulbecco's phosphate-buffered saline (PBS) (500-mL; Gibco, Carlsbad, CA; cat. no. 14190-144).
4. Gelatin (Sigma, Milwaukee, WI; cat. no. G-1890).
5. 35-mm polystyrene cell culture dishes (Corning, Acton, MA; cat. no. 430165).
6. 100 mL trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco, cat. no. 25300-054).
7. 15-mL centrifuge tubes (VWR, West Chester, PA; cat. no. 21008-103).
8. T-175 cell culture flasks (tissue culture treated with 0.2-mm vent cap; Corning, cat. no. 431080).
9. Nunc cryotube vials (VWR, cat. no. 66021-986).
10. Nalgene Cryo 1°C freezing containers (Research Products International, Mt. Prospect, IL; cat. no. 5100-0001).
11. 2 mg mitomycin C (Sigma, cat. no. M4287): for mitomycin C solution, dissolve 2.0 mg mitomycin C powder in 200 mL fibroblast feeder cell medium (10 µg/mL). Stock may be stored in the dark at 4°C for up to 6 wk or –20°C for long-term storage.

2.1.1. Media

1. High-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco, cat. no. 119650-092).
2. 500 mL heat-inactivated fetal bovine serum (FBS) (Cellgro, Herndon, VA; cat. no. 35-001-CV).
3. 100 mL penicillin-streptomycin (from 100X stock) (Gibco, cat. no. 15070-063).
4. 100 mL L-glutamine (from 100X stock) (Gibco, cat. no. 25030-081).
5. 500 mL Knockout DMEM (k-o DMEM) (Gibco, cat. no. 10829-018).
6. 500 mL ES cell-qualified FBS (Gibco, cat. no. 16141-079).
7. 500 mL Knockout serum replacement (KSR) (Gibco, cat. no. 10828-028).
8. LIF (10^6 U; Chemicon International, Temecula, CA; cat. no. ESG1106).
9. 250 mL β-mercaptoethanol (Sigma, cat. no. M-7522).
10. 100 mL minimum essential medium (MEM) nonessential amino acids (Gibco, cat. no. 11140-050).
11. 100 mL dimethyl sulfoxide (DMSO) Hybri-Max (Sigma, cat. no. D2650).
12. Embryonic fibroblast feeder cell medium (or STO cells may be used): 88% high-glucose DMEM, 10% heat-inactivated FBS, 1% penicillin-streptomycin, and 1% L-glutamine. To prepare 100 mL medium, combine 88 mL DMEM with 10 mL FBS, 1 mL penicillin-streptomycin, and 1 mL L-glutamine. This medium may be stored for up to 1 mo at 4°C.
13. ES-D3 cell medium: 78% KO-DMEM, 15% ES cell-qualified FBS, 5% KSR, 1% penicillin-streptomycin, 1% L-glutamine, 1000 U/mL of LIF, and 5×10^{-5} *M* β-mercaptoethanol. To prepare 50 mL medium, combine 38.5 mL of k-o DMEM, 7.5 mL ES cell-qualified FBS, 2.5 mL KSR, 0.5 mL penicillin-streptomycin, 0.5 mL L-glutamine, 0.5 mL nonessential amino acids, 50 µL LIF, and 10 µL diluted (35 µL β-mercaptoethanol in 5 mL PBS). ES cell medium may be stored for up to 2 wk at 4°C.

14. Freezing medium: both ES cells and fibroblast feeder cells are frozen in medium containing 50% cell culture medium and 50% freezing medium. The freezing medium is composed of 80% FBS and 20% DMSO and should be made up fresh for each use.

2.1.2. ES-to-EC Cell Differentiation

1. Collagen IV Biocoat Cellware 35-mm dishes (Becton-Dickinson, Franklin Lakes, NJ; cat. no. 354459).
2. 500 mL α-MEM (Gibco, cat. no. 12561-056).
3. 500 mL heat-inactivated FBS (Cellgro, cat. no. 35-001-CV).
4. 250 mL β-mercaptoethanol (Sigma, cat. no. M-7522).
5. 100 mL penicillin-streptomycin (from 100X stock) (Gibco, cat. no. 15070-063).
6. 100-mL bottle L-glutamine (from 100X stock) (Gibco, cat. no. 25030-081).
7. ES cell differentiating medium: 93% α-MEM, 5% FBS, 1% penicillin-streptomycin, 1% L-glutamine, and 5×10^{-5} *M* β-mercaptoethanol. To prepare 50 mL medium, combine 44 mL α-MEM, 5 mL FBS, 0.5 mL penicillin-streptomycin, 0.5 mL L-glutamine, and 10 µL diluted β-mercaptoethanol (35 µL β-mercaptoethanol in 5 mL PBS). ES cell differentiating medium may be stored for up to 1 mo at 4°C.

2.1.3. Purification of Vascular Progenitor Cells

1. 100 mL cell dissociation solution (Sigma, cat. no. C-5914).
2. 500 mL heat-inactivated FBS (Cellgro, cat. no. 35-001-CV).
3. 500 mL Dulbecco's PBS (Gibco, cat. no. 14190-144).
4. 25 g Cohn analog bovine albumin (Sigma, cat. no. A-1470): add 0.6 g bovine albumin to 200 mL PBS to make 0.3% bovine serum albumin (BSA) buffer solution. Place this mixture in the water bath until the albumin is dissolved (sterile filter the solution if needed).
5. Rabbit anti-mouse Flk-1 (Alpha Diagnostic International, San Antonio, TX; cat. no. FLK11-A).
6. Conjugated donkey anti-rabbit phycoerythrin (PE) (Research Diagnostics Inc., Flanders, NJ; cat. no. RDI-711116152).
7. 10 mL normal donkey serum (Research Diagnostics Inc., cat. no. RDI-NSDNKY).
8. 5-mL sterile polystyrene round-bottom tubes (Becton-Dickinson, cat. no. 352058).
9. Collagen IV Biocoat Cellware 35-mm dishes (Becton-Dickinson, cat. no. 354459).
10. Recombinant human $VEGF_{165}$ (R&D Systems, Minneapolis, MN; cat. no. 293-VE).
11. ES differentiating medium (*see* **Subheading 2.1.2.**, **item 7**).

2.1.4. Purification and Expansion of ECs From Vascular Progenitor Cells

1. Aspirator assembly (5 pack; Sigma, cat. no. A5177).
2. Acrodisc syringe filter (0.2-µm membrane; Pall, East Hills, NY; cat. no. 4192).
3. Glass Pasteur pipets (9 in.; VWR, cat. no. 53283-915).
4. Autoclavable pipet tips (200–1000 µL; VWR, cat. no. 53508-819).
5. 100-mL bottle cell dissociation solution (Sigma, cat. no. C-5914).
6. Endothelial growth medium (EGM)-2 medium Bullet Kit (500-mL bottle plus growth factors; Clonetics, East Rutherford, NJ; cat. no. CC-3162).
7. Recombinant human $VEGF_{165}$ (R&D Systems, cat. no. 293-VE).
8. 1 mg collagen IV (Becton-Dickinson, cat. no. 354233).
9. 100 mg collagen I (Becton-Dickinson, cat. no. 354236).
10. 2 mg fibronectin (Sigma, cat. no. F-1141).
11. Gelatin (Sigma, cat. no. G-1890).

2.1.5. General Equipment (see **Note 1**)

1. Water bath at 37°C.
2. Coulter counter or hemocytometer for cell counting.
3. Inverted microscope with ×5 and ×10 objectives.
4. Humidified incubator at 37°C and 5% CO_2.
5. Laminar flow cabinet.
6. Liquid nitrogen storage tank.
7. Micropipets and tips (10, 20, 100, 200, and 1000 µL).
8. Clean hood.
9. Stereomicroscope.
10. Disposable pipets (1, 5, 10, 25, and 50 mL).
11. Refrigerator (4°C) and freezer (–20 and –80°C).
12. Tabletop centrifuge.

2.2. Immunofluorescent Staining

1. Tissue culture-treated four-well glass chamber slides (Becton-Dickinson, cat. no. RF354114).
2. Lab-Tek Permanox four-well chamber slides (Becton-Dickinson, cat. no. 177437).
3. Gelatin (Sigma, cat. no. G-1890).
4. 100 mg rat tail type I collagen (Becton-Dickinson, cat. no. 354236).
5. PBS (Gibco, cat. no. 14190-144).
6. 25 g Cohn analog bovine albumin (Sigma, cat. no. A-1470): for 0.3% BSA buffer, use 0.6 g bovine albumin and 200 mL PBS. Make up fresh for each use.
7. 10 mL normal donkey serum (Research Diagnostics Inc., cat. no. RDINSDNKY).
8. 100 mg Hoechst 33258 nucleic acid stain (Molecular Probes, Eugene, OR; cat. no. H1398).
9. 20% aqueous formaldehyde (Tousimis Research Corp., Rockville, MD; cat. no. 1008A). For 4% formaldehyde solution in PBS, use 10 mL Tousimis 20% formaldehyde and 40 mL PBS. Store for up to 1 mo in the dark at 4°C.
10. 500-mL bottle Triton X-100 (US Biochemical Corp., Cleveland, OH; cat. no. 22686). For 0.5% Triton solution, use 5 mL Triton in 1 L PBS and stir for 10 min. This may be kept on the lab bench.
11. Aqueous mounting medium S3025 (Dako Corp., Carpinteria, CA; cat. no. 002972).
12. Micro cover glasses (cover slips; VWR, cat. no. 48393 252).
13. Alkaline phosphatase (Leukocyte Kit, Sigma, cat. no. 86R).
14. Rabbit anti-Oct4 (H-134; Santa Cruz Biotechnology, Santa Cruz, CA; cat. no. sc-9081).
15. Rat anti-mouse E-cadherin (ECCD-2; Zymed Laboratories, South San Francisco, CA; cat. no. 13-1900).
16. Fluorescein isothiocyanate (FITC)-conjugated mouse anti-stage-specific embryonic antigen 1 (Santa Cruz Biotechnology, cat. no. sc-21702).
17. PE-conjugated donkey anti-rabbit immunoglobulin (Ig) G (Research Diagnostics, cat. no. RDI-711116152).
18. FITC-conjugated mouse anti-rat IgG_{2a} (Pharmingen, San Diego, CA; cat. no. 10094D).
19. FITC-conjugated donkey anti-rat IgG (Research Diagnostics, cat. no. RDI-712095150).
20. Rabbit anti-Flk-1/KDR (Alpha Diagnostic International, cat. no. FLK11-A).
21. Rabbit anti-human Flt-1 (Alpha Diagnostic International, cat. no. FLT11-A).
22. Biotin-conjugated rat anti-mouse PECAM-1 (MEC13.3; Pharmingen, cat. no. 1952D).
23. FITC-conjugated rat anti-mouse PECAM-1 (MEC13.3; Research Diagnostics, cat. no. RDI-mCD31abrt-FT).
24. FITC-conjugated rat anti-mouse CD34 (RAM34; PharMingen, cat. no. 553733).

25. Rat anti-mouse VE-cadherin (11D4.1; Pharmingen, cat. no. 555289).
26. Rat anti-mouse CD105/endoglin (MJ7/18; Southern Biotechnology, Birmingham, AL; cat. no. 1860-01).
27. PE-conjugated donkey anti-rabbit IgG (Research Diagnostics, cat. no. RDI-711116152).
28. Streptavidin cy-chrome (Pharmingen, cat. no. 13038A).
29. FITC-conjugated mouse anti-rat IgG_{2a} (Pharmingen, cat. no. 10094D).
30. FITC-conjugated donkey anti-rat IgG (Research Diagnostics, cat. no. RDI-712095150).

3. Methods

3.1. ES Cell Culture (see Note 2)

*3.1.1. Freezing Cells (see **Note 3**)*

1. Trypsinize cells in the exponential phase of growth (varies for each cell type but typically is after 3 d of growth).
2. Pellet the cells by centrifugation (200*g*, 4–5 min) and resuspend in an appropriate amount of cell culture medium (*see* **Note 4**).
3. Slowly add the same amount of the freezing medium (dropwise) over 2 min. Continuously shake the cell suspension for even distribution of the DMSO/FBS freezing medium.
4. Aliquot 1 mL cell suspension into cryovials.
5. Immediately transfer cryovials to a Cryo 1°C freezing container and place the container in a –70°C or a –80°C freezer for 24 h.
6. Transfer the vials to liquid nitrogen.

3.1.2. Thawing Cells

1. Thaw the cells in a 37°C water bath until only a small ice droplet remains (approx 1 min; the last drop will thaw as you carry the vial to the laminar flow cabinet).
2. While the vial is thawing, fill a 15-mL centrifuge tube with 10 mL of the appropriate cell culture medium.
3. Transfer the thawed cells to the centrifuge tube and collect the cells by centrifuging at 200*g* for 4–5 min.
4. Remove the supernatant and gently resuspend the cells in fresh growth medium.
5. Transfer all cells to the prepared culture dish (*see* **Note 5**) and place in a 37°C incubator.
6. Replace the medium with fresh medium the next day.

3.1.3. Inactivation of Fibroblast Feeder Cells

Typically, ES cells are cultured on fibroblast feeder cells that are inactivated with mitomycin C or irradiation. The inactivation of the fibroblast cells allows the ES cells to benefit from the co-culture feeder conditions without fibroblast proliferation (*see* **Notes 6** and **7**).

1. After thawing, allow mouse fibroblasts to grow to 90–95% confluency in T-175 tissue culture-treated flasks.
2. Aspirate culture medium from the flask and replace with 16 mL mitomycin C solution.
3. Incubate the treated flasks for 2 h at 37°C and 5% CO_2.
4. After 2 h, aspirate mitomycin C solution and wash each T-175 flask five times with 20 mL PBS.
5. Add 3 mL trypsin per flask and monitor cell detachment. After approx 1 min, cells should detach from the flask surface (gently rock flask side to side).
6. After cells have detached, add 5–10 mL fibroblast feeder cell medium.

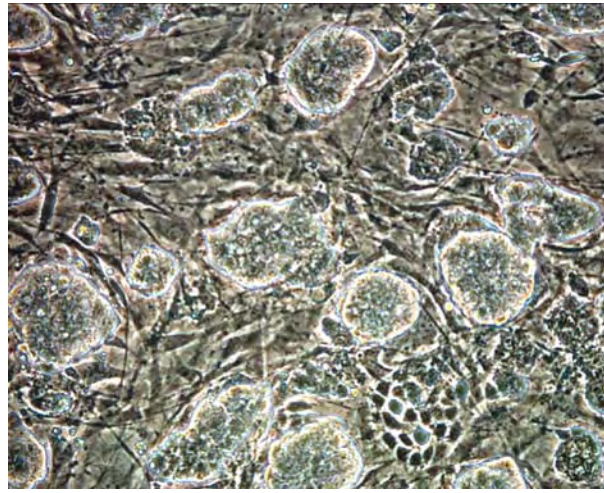

Fig. 1. Mouse embryonic stem (ES) cell colonies on embryonic fibroblast feeder cells. Note that, in general, the ES cell colonies are not in contact with one another; however, within 1 d these cell colonies will continue to expand radially outward. Therefore, these cells should be subcultured within the day.

7. Transfer the cell suspension from each flask to 15-mL centrifuge tubes.
8. Centrifuge at 200*g* for 4–5 min.
9. Remove supernatant and wash again with 10 mL fibroblast feeder cell medium per tube.
10. Centrifuge at 200*g* for 4–5 min.
11. Repeat washing one more time.
12. Count cells.
13. Plate between 3×10^5 and 4×10^5 cells per 35-mm dish that will be needed for ES cells. Fill dishes with 2.5 mL fibroblast feeder cell medium each; allow at least 4 h, preferably overnight, for the cells to adhere to dishes before adding ES cells (*see* **Note 8**).

3.1.4. Culture of ES Cells

1. Thaw D3-ES cells as described in **Subheading 3.1.2.**; make sure to use mouse ES cell medium.
2. Plate 1×10^5 to 5×10^5 D3-ES cells per fibroblast feeder cell-coated 35-mm dish with 2.5 mL mouse ES cell medium.
3. Replace culture medium daily.
4. The cells will need to be subcultured before colonies begin to touch (*see* **Note 9** and **Fig. 1**). If you plate 2.5×10^5 cells per 35-mm dish, then they will need to be subcultured in 3 d.

To subculture ES cells:

5. Gently remove culture medium and rinse cells twice with 3 mL PBS per 35-mm dish.
6. Add 1 mL trypsin per 35-mm dish and place cells in the incubator for 1–2 min.
7. Gently pipet cells up and down 10 times to disaggregate cells.
8. Add 3 mL mouse ES cell medium and transfer all of the cell suspension to a 15-mL centrifuge tube.

9. Add 3–5 mL more mouse ES cell medium to completely neutralize the trypsin.
10. Again, gently pipet cells up and down 10–30 more times to disaggregate cells (*see* **Note 10**).
11. Count cells.
12. Centrifuge at 200*g* for 4–5 min.
13. Remove supernatant.
14. Resuspend pellet in appropriate quantities of mouse ES cell medium and replate at 1×10^5 to 5×10^5 D3-ES cells per fibroblast feeder cell-coated 35-mm dish with 2.5 mL mouse ES cell medium per dish.

3.2. ES-to-EC Cell Differentiation

The cells are subcultured on 0.1% gelatin (no feeders) for 3–6 d before switching to differentiation conditions. This allows expansion of the ES cells while minimizing the number of feeder cells in the culture.

1. Gently remove culture medium and rinse cells twice with 3 mL PBS per 35-mm dish.
2. Add 1 mL trypsin per 35-mm dish and place cells in the incubator for 1–2 min.
3. Gently pipet cells up and down 10 times to disaggregate cells.
4. Add 3 mL ES-to-EC cell differentiating medium and transfer all of the cell suspension to a 15-mL centrifuge tube.
5. Add 3–5 mL more ES-to-EC cell-differentiating medium to completely neutralize the trypsin.
6. Again, gently pipet cells up and down 10–30 more times to disaggregate cells.
7. Count cells.
8. Centrifuge at 200*g* for 4–5 min.
9. Remove supernatant.
10. Resuspend pellet in 1 mL ES-to-EC cell differentiating medium and gently pipet cells up and down 10–30 more times to disaggregate cells.
11. Add 2.5 mL ES-to-EC cell differentiating medium each to 2–4 collagen IV Biocoat Cellware 35-mm dishes.
12. Add 30,000 cells (calculated volume) to each 35-mm collagen IV-coated 35-mm dish.
13. Incubate at 37°C and 5% CO_2 for 4 d. Do not change culture medium during these 4 d.

3.3. Purification of Vascular Progenitor Cells

After 4 d of differentiation, the ES cells will consist of a heterogeneous mixture of progenitor cells (*see* **Note 11** and **Fig. 2**). Included in the mixture will be a population of Flk-1-expressing cells that are vascular progenitor cells (for discussion, *see* **refs. *8–10*** and ***12***) and blood precursor cells. Using flow cytometry, the brightest Flk-1-expressing cells can be isolated from the heterogenous mixture of cells.

1. Remove culture medium and wash cells twice with 3 mL PBS per 35-mm dish.
2. Add 3 mL cell dissociation solution each and allow cells to incubate at 37°C for 20–30 min (*see* **Note 12**).
3. Pipet up and down 10 times while washing solution over the bottom of the dish to remove all the cells. If some cells still adhere to the bottom of the dish, then use a cell scraper to remove the remaining cells.
4. Transfer cells to a 15-mL centrifuge tube and add 3 mL FBS.
5. Centrifuge cells at 200*g* for 4–5 min. The cells are now ready for immunostaining (*see* **Note 13**).
6. Chill the buffer solution at 4°C or keep buffer solution on ice.
7. Remove supernatant from the cell pellet.

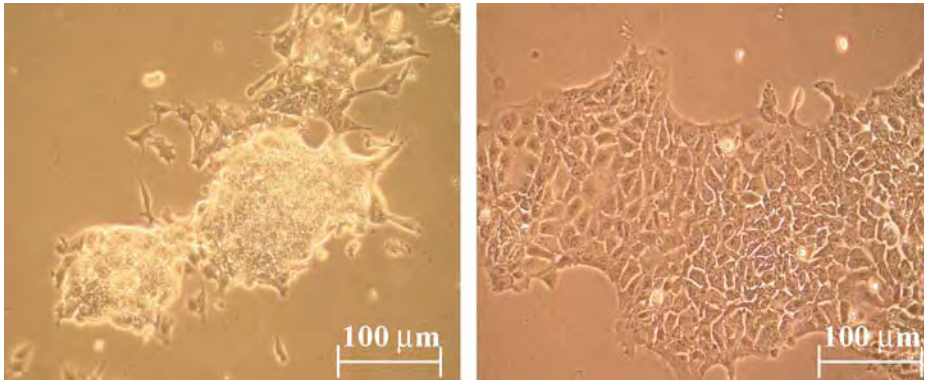

Fig. 2. Mouse embryonic stem (ES) cell colonies on gelatin (**left**). Mouse ES cells after 4 d of differentiation on collagen type IV (**right**). Note the distinct changes in morphology between undifferentiated ES cells and differentiated ES cells.

8. Resuspend the entire cell pellet in 1 mL BSA buffer solution and 10% donkey serum.
9. Incubate on ice or at 4°C for 1 h.
10. Add 4 mL BSA buffer solution and centrifuge at 200*g* for 4–5 min.
11. Remove aspirate and resuspend the cell pellet in 400 µL BSA buffer solution. Pipet up and down to evenly distribute the cells in the solution.
12. Place 50 µL cell suspension in another centrifuge tube and label it "cells only."
13. Place 50 µL cell suspension in another centrifuge tube and label it "PE only."
14. Label the original cell suspension "Flk-1 PE."
15. Add 250 µL BSA buffer to the two new centrifuge tubes. All tubes should now be at 300 µL.
16. Add 8 µL Flk-1 antibody to the Flk-1 PE tube.
17. Incubate all tubes on ice or at 4°C for 30 min.
18. Add 4 mL BSA buffer solution to all tubes and centrifuge at 200*g* for 4–5 min.
19. Remove aspirate and resuspend the cell pellets in 300 µL BSA buffer solution each. Pipet up and down to evenly distribute the cells in the solution.
20. Add 8 µL donkey anti-rabbit PE to the Flk-1 PE and PE only tubes (*see* **Note 14**).
21. Incubate all tubes on ice or at 4°C for 30 min.
22. Add 4 mL BSA buffer solution to all tubes and centrifuge at 200*g* for 4–5 min.
23. Remove aspirate and repeat **step 22**.
24. Resuspend the cells in the Flk-1 PE tube in 1 mL BSA buffer and transfer the cell solution to a labeled 5-mL round-bottom polystyrene fluorescent-activated cell sorting (FACS) tube.
25. Resuspend the cells in the cells only and PE only tubes in 300 µL BSA buffer and transfer the cell solutions to labeled 5-mL round-bottom polystyrene FACS tubes.
26. Fill a fourth 5-mL round-bottom polystyrene FACS tube with 1.5 mL ES-to-EC cell differentiating medium. This will serve as your collection tube for FACS.
27. Sort the brightest population of Flk-1-expressing cells into the collection tube (*see* **Note 15** and **Fig. 3**).
28. Centrifuge the collection tube containing Flk-1-positive cells at 200*g* for 3–4 min.
29. Remove aspirate and resuspend cells in 1 mL ES-to-EC cell differentiating medium. Based on number of cell-sorting events, calculate the volume of cell suspension to add to each 35-mm collagen IV-coated dish (you will want approx 50,000–100,000 cells per dish).

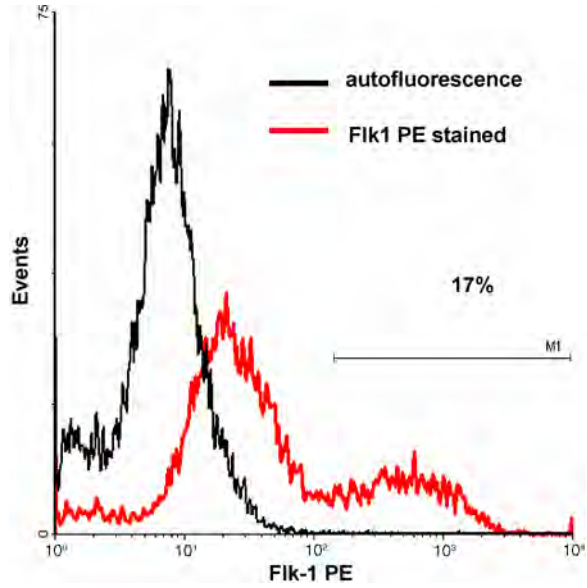

Fig. 3. Some embryonic stem cells exhibit high Flk-1 expression after 4 d on collagen type IV. These are the vascular progenitor cells to isolate from the rest of the heterogenous mixture of cells.

30. Fill each dish to a total volume of 2.5 mL ES-to-EC cell differentiating medium.
31. Add 125 µL VEGF (50 ng/mL) to each dish.
32. Put the cells in a 37°C incubator and do not move dishes for 4 d.
33. On d 4, aspirate off old medium and add 2.5 mL fresh ES-to-EC cell differentiating medium per 35-mm dish (*see* **Note 16**).

3.4. Purification of ECs From Vascular Progenitor Cells

After culturing for approx 1 wk, the Flk-1-positive cell outgrowths exhibit predominantly two different morphologies (*see* **Fig. 4**). These include endothelial-like cells with a cobblestone morphology and more striated smooth muscle-like cell populations. Because these two populations are distinctly different in appearance, it is possible to manually isolate the ECs and replate them in clean dishes for further purification.

3.4.1. Pipet Preparation

1. Hold the two ends of a Pasteur pipet in a low flame until the pipet is hot. This heated portion should be close to the tip of the Pasteur pipet.
2. Quickly pull on the tip of the pipet while lifting the pipet out of the flame. This will generate a pipet region with a smaller diameter just above the tip of the pipet.
3. Loop back the pulled glass and rub glass to glass to create a point of friction. Tap the glass to break the tip off at the point of friction (*see* **Note 17**).
4. Polish the new end of the pipet by passing the new tip gently over a low flame (*see* **Note 18**).

3.4.2. Assemble Mouth Aspirator

To assemble the mouth aspirator, fit the narrow end of a 1000-mL micropipet tip into the rubber tubing of an aspirator assembly fitted with a 0.2-µm syringe filter. The Pasteur

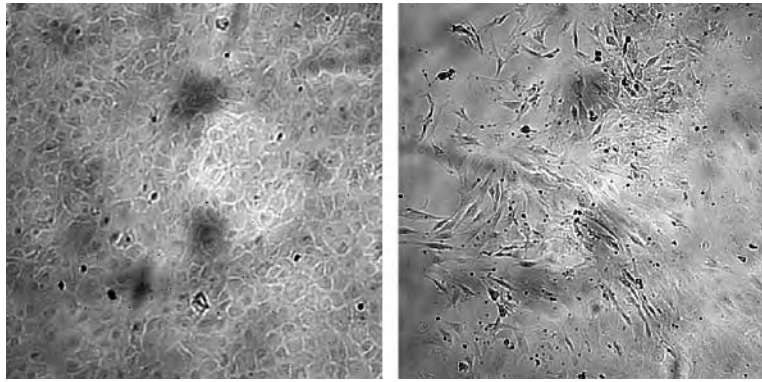

Fig. 4. Outgrowths of Flk-1-positive cells consisted of primarily two cell populations: endothelial-like cells exhibiting a cobblestonelike morphology (**left**) and striated smooth muscle-like cells (**right**). The smooth muscle-like cells were positive for α-smooth muscle actin (data not shown).

pipet now fits into the wide end of the 1000-µL micropipet tip. This aspirator assembly allows for simultaneous microscope viewing and cell colony manipulations.

3.4.3. Manual Isolation of EC Cells

1. Aspirate culture medium and wash cells twice with 3 mL PBS per 35-mm dish.
2. Incubate cells with cell dissociation solution for 5 min (*see* **Note 19**).
3. Meanwhile, fill 6–10 collagen IV-coated 35-mm dishes with 2 mL EC medium.
4. Carve around a 5- to 10-cell cluster with the edge of the mouth-Pasteur pipet assembly.
5. Aspirate the cells into the pipet and transfer to the new 35-mm dishes containing 2 mL ES-EC differentiation medium (*see* **Fig. 5**).
6. Repeat carving out another 5- to 10-cell cluster of cells and plate in a new 35-mm dish.
7. Repeat for at least 6–10 dishes.
8. Add 50 ng/mL VEGF to each dish.
9. Incubate cells at 37°C and 5% CO_2 for 7–10 d.

3.5. In Vitro Expansion of ECs

1. After allowing the cells 7–10 d of uninterrupted growth, observe the dishes carefully for EC colonies. Once the cell colonies are well established, you will see 50–100 cells in a circular sheet. These cells will be highly confluent in the center and appear to grow outward at the edges of the colony.
2. To encourage further cell proliferation, the cells should be subcultured in EC medium to allow cells to grow easily on the entire surface of the collagen IV-coated 35-mm dish (*see* **Fig. 6**).
3. The cells should then be continually expanded in larger dishes (35-mm dish, then use T-25 flasks, then use T-75 flasks, then use T-175 flasks, and then use multiple T-175 flasks) (*see* **Note 20**).

3.6. Immunostaining

The following protocol may be used for antibodies against both ES cells and ES-derived ECs (*see* **Note 21**).

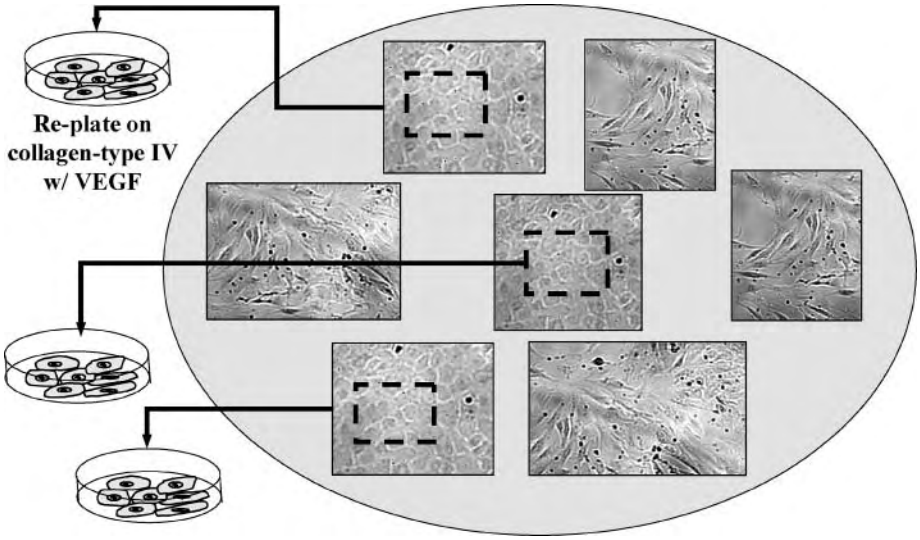

Fig. 5. Endothelial-like cells exhibiting a cobblestonelike morphology are manually picked based on proper morphology and replated on a second dish coated with collagen IV. Note that several batches of endothelial cells may be isolated from one dish. These batches may vary slightly, so it is a good idea to expand the batches separately.

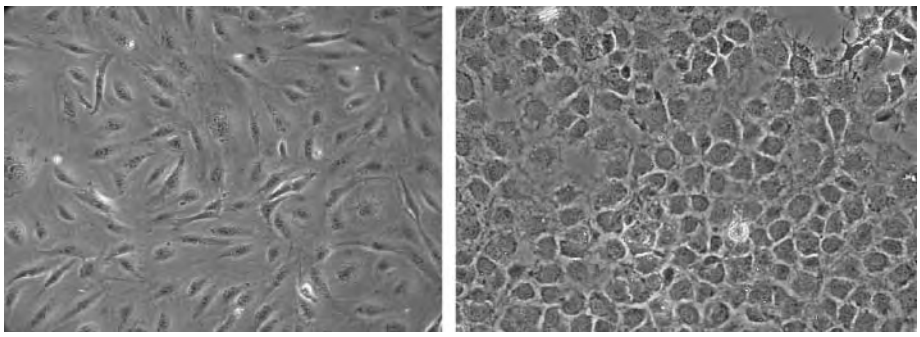

Fig. 6. Embryonic stem-derived endothelial cells at low confluence (**left**) and high confluence (**right**).

3.6.1. Coating Chamber Slides

1. Coat four-well chamber slides with collagen IV, collagen I, fibronectin, or gelatin for 2 h prior to cell seeding.
2. Wash with PBS.

3.6.2. Seeding Cells on Slides

1. To coated chamber slides, add 1 mL EC medium containing 10,000 ES-derived ECs into each well.

2. Allow the cells to grow to confluence.
3. Wash cells twice with PBS.
4. Fix the sample in 4% formaldehyde solution for 5–10 min at room temperature.
5. Wash cells twice with PBS and store at 4°C until ready to stain (*see* **Note 22**).
6. Coat the cells with 0.5% Triton.
7. Incubate 5–10 min to permeabilize the cell membrane.
8. Wash with 0.3% BSA buffer.
9. Incubate the fixed cells with 0.3% BSA buffer containing 5–10% normal donkey serum for 1 h to block.
10. Wash with 0.5% Triton.
11. Dilute the primary antibodies for staining in 1:50 to 1:200 in 0.3% BSA buffer and place the staining solution on the slide. Allow the samples to incubate at room temperature for 30 min.
12. Wash all samples twice with 0.5% Triton.
13. Dilute the secondary antibody between 1:50 and 1:200 in 0.3% BSA and add 20 µg/mL Hoechst to counterstain the cell nucleus; incubate samples at room temperature for 30 min.
14. Wash twice with 0.3% BSA buffer.
15. Remove the wells from the microscope slide and cover the microscope slide with aqueous mounting medium and a cover slip with no trapped air. Apply nail polish around all of the edges of the cover slip to keep the cells from drying (*see* **Note 23**).

4. Notes

1. All cells must remain sterile throughout passaging and differentiation procedures. This means paying special attention to sterile techniques and always using sterile culture dishes, pipets, and the like. Also, warm all media solutions to 37°C before use.
2. The ES culturing methods described will provide approx 10^6 ES cells per 35-mm dish at confluence. These small dishes are maintained because of the expense of reagents; however, if larger numbers of cells are desired, then this protocol may be scaled up proportionally, keeping constant the cell seeding density (the number of cells per square centimeter).
3. Generally, freeze cells slowly, and thaw cells quickly. For long-term storage, cells must be kept in liquid nitrogen.
4. For convenience, cells are frozen in 1-mL aliquots at cell numbers that correspond to the appropriate numbers that will be needed on thawing. The upper limit would be $5–10 \times 10^6$ cells/mL.
5. Both ES and EC cells are maintained on dishes or flasks coated with the appropriate substrate; therefore, when thawing or passaging cells, make sure to allow time (1–2 h) for the substrate to adhere to the culture dish and wash off excess substrate with PBS. For ES cells that will be cultured on fibroblasts, make sure to prepare those dishes with a layer of fibroblast cells at least 4 h prior to ES cell seeding.
6. Mouse embryonic fibroblast feeder cells are typically used; however, the isolation of these cells requires several animals to be sacrificed and labor-intensive dissection of the fetal tissue. If mouse embryonic feeders are unavailable or undesirable, then STO cells may also be used (available from ATCC).
7. Before disposal, mitomycin C must be neutralized with Clorox bleach for at least 15 min.
8. Inactivated fibroblasts may be used for up to 1 wk.
9. ES cells maintain their undifferentiated state best when the colonies are subcultured before the colonies contact other colonies.
10. We want a single-cell suspension, and fibroblasts tend to stick together. Therefore, allow 2 min for the large cell clumps to sink to the bottom of the centrifuge tube, transfer the top

three-quarters of the cell suspension to another centrifuge tube, and discard the fibroblast cell clumps. This technique also ensures that fewer fibroblasts are subcultured in the next dish.
11. When ES cells begin to differentiate, they will lose the typical three-dimensional colony appearance and begin to grow more like monolayer cell cultures.
12. When staining cells for extracellular surface markers, it is very important to use a nonenzymatic method for removing the cells from the culture dishes; therefore, do not use trypsin when staining cells. Trypsin will degrade the surface markers that you are attempting to stain.
13. Take care to keep the cells sterile during the entire staining and sorting procedures. All solutions for staining will be kept at 4°C or on ice.
14. Fluorescent antibodies should be kept in the dark during storage and when labeling cells. Exposure to too much light may cause the fluorescent molecules to emit light prematurely.
15. Usually, there will be a subpopulation of cells that is expressing a very high number of Flk-1 surface molecules. This population will be the "brightest" population of cells falling in the highest channels of your FACS histogram. This population of cells typically ranges from 10 to 30% of your total cell population.
16. Most of these cells will die because of staining and FACS procedures. Do not move the dishes or change medium for 4 d and then allow at least 1 wk before expecting to see any cell growth.
17. The technique for pulling Pasteur pipets will take some practice.
18. The Pasteur pipets should remain sterile, so use immediately after pulling or pull several pipets and autoclave ahead of time.
19. As cells begin to detach from the culture dish, their distinct cell morphologies may become vague; therefore, it is helpful to mark the bottom of the dish with the appropriate location of the desired cells.
20. Make sure to coat the surface of each flask with collagen IV, collagen I, fibronectin, or gelatin for 2 h prior to cell seeding and wash off the extra substrate with PBS. The ES cell-derived ECs can be frozen and thawed normally.
21. For color images of stained cells, *see* **ref.** ***12***.
22. Store on wet paper towels or wrap in parafilm to keep from dehydrating.
23. Stored in the dark, the cells should remain fluorescent for up to 1 wk.

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21

Differentiation of Mouse Embryonic Stem Cells Into Endothelial Cells

Genetic Selection and Potential Use In Vivo

Clotilde Gimond, Sandrine Marchetti, and Gilles Pagès

Summary

Embryonic stem (ES) cells are pluripotent cells derived from blastocyst-stage embryos. They are characterized by their infinite self-renewal capacity and their ability to differentiate into many cell types in vitro as well as in vivo. The present protocol describes culture conditions that allow efficient differentiation of mouse ES cells toward the endothelial lineage, both in two-dimensional cultures and in three-dimensional, multicellular embryoid bodies. We also provide a protocol for establishing recombinant ES cell clones, giving the example of cells expressing green fluorescent protein and an antibiotic resistance gene under the control of an endothelial-specific promoter. These transgenes allow the visualization and the genetic selection of endothelial cells from a heterogeneous population of differentiating ES cells. Potential applications for these differentiation models are given, including the study of the endothelial cell lineage and its derivatives, the testing of molecules involved in angiogenesis, and the potential use of selected endothelial cells in vivo.

Key Words: Angiogenesis; differentiation; embryoid body; endothelial cell transplantation; endothelial cells; lineage selection; mouse embryonic stem cells; smooth muscle cells.

1. Introduction

Embryonic stem (ES) cells are pluripotent cells derived from the inner mass of blastocyst stage embryos. In the past 20 yr, they have proved to be immensely valuable for the study of cell differentiation and tissue development, both in vitro and in whole organisms. This is not only because of their pluripotency, but also because of their infinite self-renewal capacities and the simultaneous development of homologous recombination and transgenesis technology. In vitro, ES cells can differentiate into a variety of cell types, including cardiac, epithelial, neuronal, hematopoietic, or endothelial cells (ECs), when appropriate factors or culture conditions are provided. Endothelial differentiation is strongly promoted by angiogenic factors such as vascular endothelial

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growth factor (VEGF) or fibroblast growth factor (FGF) and can occur in both two-dimensional cultures and three-dimensional embryoid bodies (EBs) formed on ES cell aggregation.

In vitro models for EC differentiation may be used to: (1) study the role of external factors on EC growth and differentiation, including soluble growth factors, matrix proteins, or other cell types present in the culture; (2) define the role of a given protein in EC differentiation, for example, by gene invalidation; (3) test the activity of putative novel angiogenic factors or, in contrast, the activity of angiogenesis inhibitors, including novel drugs; and (4) purify ECs in large numbers in the context of cell therapy to improve postischemic revascularization.

1.1. Differentiation of ES Cells in EBs

There are several methods to induce the formation of EBs. In our laboratory, we induce their formation by aggregating ES cells in hanging drops, using gravity as an aggregation force, as described in Mummery et al. ***(1)***. This method presents the advantage of obtaining well-calibrated EBs almost identical in size (*see* **Fig. 1**).

When grown as EBs, ES cells spontaneously differentiate into many cell types, including cardiomyocytes. ES cells may differentiate into cells from the EC lineage in unsupplemented conditions, but many will generally remain as endothelial progenitors, positive for early markers, such as CD31, also known as platelet-endothelial cell adhesion molecule (PECAM)-1 (*see* **Fig. 1C**) and vascular endothelial growth factor receptor (VEGFR)-2, but usually negative for markers occurring later in the differentiation, such as vascular endothelial cadherin (VE-cadherin) or the Tie-1 receptor. The addition of angiogenic factors in the medium, such as the FGF-2 and the more endothelium-specific VEGF (or VEGF-A or VEGF-165) efficiently promotes the differentiation of ECs and their organization into vascular structures (*see* **Fig. 1D–F**), which may contain hematopoietic cells.

Importantly, in vitro differentiation of mouse ES cells was shown to recapitulate the major steps of endothelial differentiation in the mouse embryo, with the early markers CD31 and VEGFR-2 expressed in endothelial progenitor colonies as well as in more mature structures, whereas VE-cadherin and Tie-1 expression started later in the differentiation. **Figure 1F** shows that differentiated, "vessel-like" structures express VE-cadherin. Moreover, recombinant ES cells carrying the Tie-1-GFP (green fluorescent protein) transgene show that the Tie-1 promoter is transcriptionally active in VEGF-induced vessels, as evidenced by the presence of GFP in the $CD31^+/VEGFR\text{-}2^+$ structures, while it is not active or is weakly active in $CD31^+$ colonies of endothelial progenitors (*see* **Fig. 2** and **ref.** ***2***).

Numerous studies have shown that pseudovascular structures that form in EBs derived from mouse, monkey, and human ES cells are positive for various endothelial markers, including CD31, CD34, VEGFR-1, VEGFR-2, VE-cadherin, Tie-1, and Tie-2 ***(2–8)***. It should be noted, however, that the kinetics of VEGFR-2 expression is different in monkeys and humans because it is expressed in undifferentiated ES cells, downregulated at the beginning of ES cell differentiation, and upregulated again in endothelium progenitors ***(6,8)***.

Interestingly, different angiogenic factors may induce the formation of pseudovascular structures with different shape, length, and organization. Although VEGF generally

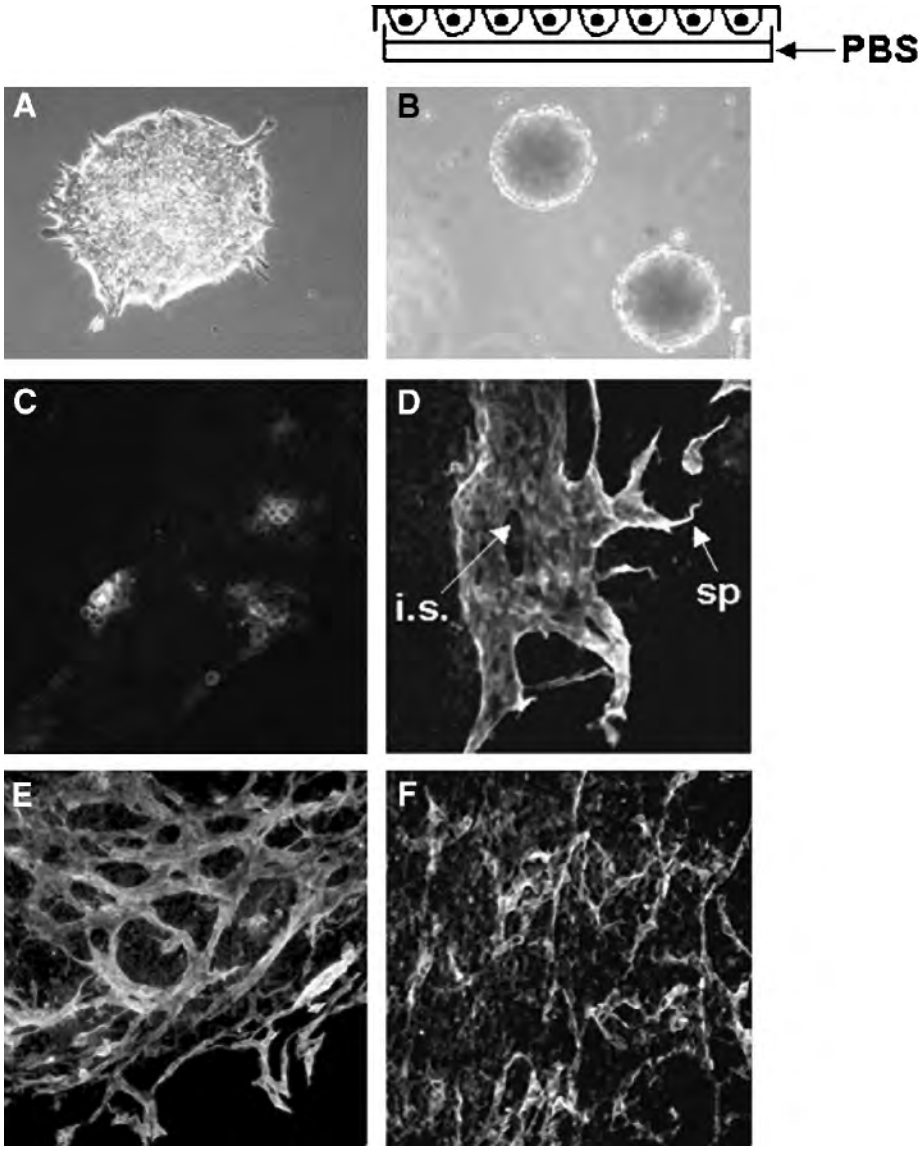

Fig. 1. Endothelial cells differentiation in embryonic stem (ES)-derived embryoid bodies. Undifferentiated ES cells (**A**) are aggregated into well-calibrated embryoid bodies (EBs) following the hanging drop method (**B**). In the absence of angiogenic growth factors, most $CD31^+$ cells remain as compact colonies inside the EB, which likely represent colonies of endothelial progenitors (**C**). In the presence of VEGF, $CD31^+$ cells further differentiate and organize into vascular trees around d 6 (**D**), which on intussusceptive (i.s.) and sprouting ("sp") angiogenesis develop into an extensive network of vessel-like structures (**E**). Vessels are also positive for a late marker of endothelial differentiation, VE-cadherin (**F**).

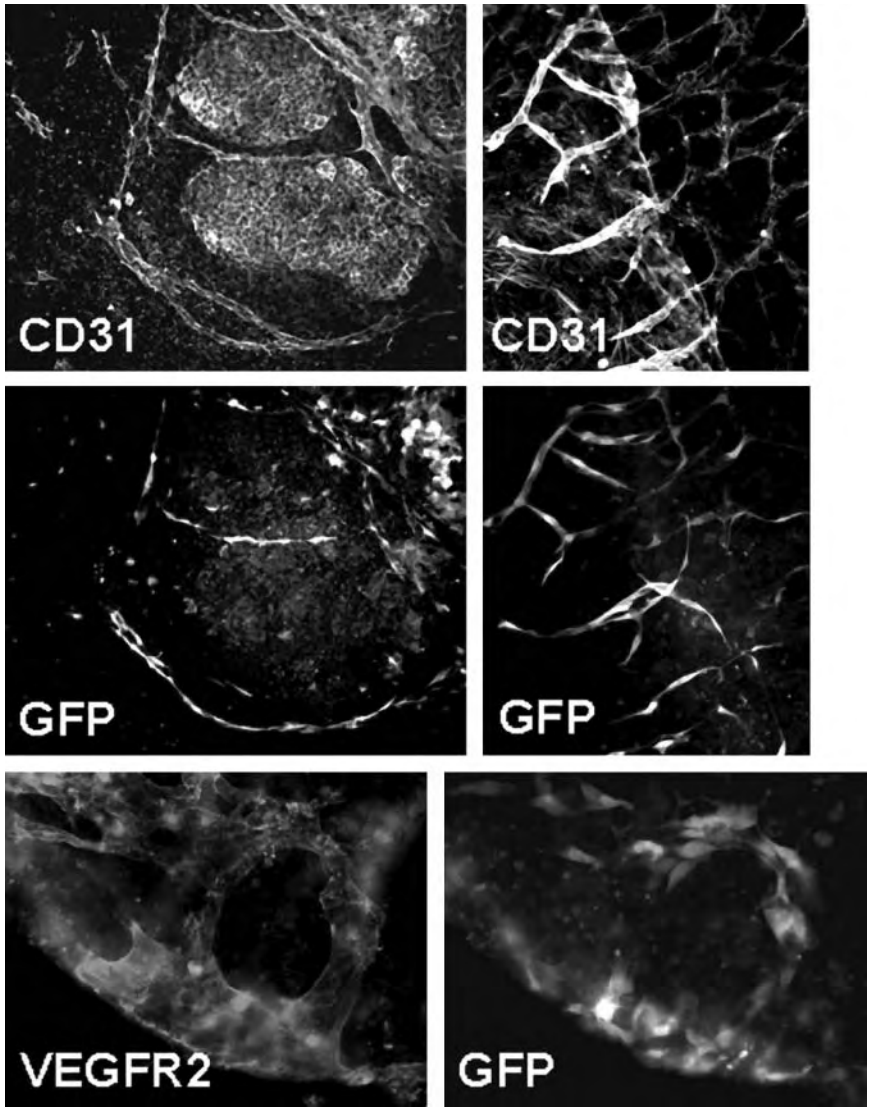

Fig. 2. Endothelium-specific expression of green fluorescent protein (GFP). Recombinant embryonic stem cells were generated that express GFP under the control of the promoter of Tie-1, a late marker of endothelial differentiation, and differentiate in the presence of vascular endothelial growth factor (VEGF). At d 7 of differentiation, vessel-like structures are positive for both CD31 and GFP, whereas round colonies of endothelial progenitors express CD31 but not yet GFP (**top left panel**), indicating that the Tie-1 promoter is not transcriptionally active in progenitors. At d 10 of differentiation, all vessels expressing GFP are also positive for CD31 (**top right panel**). The same GFP^+ structures also express VEGFR2 (**bottom panel**).

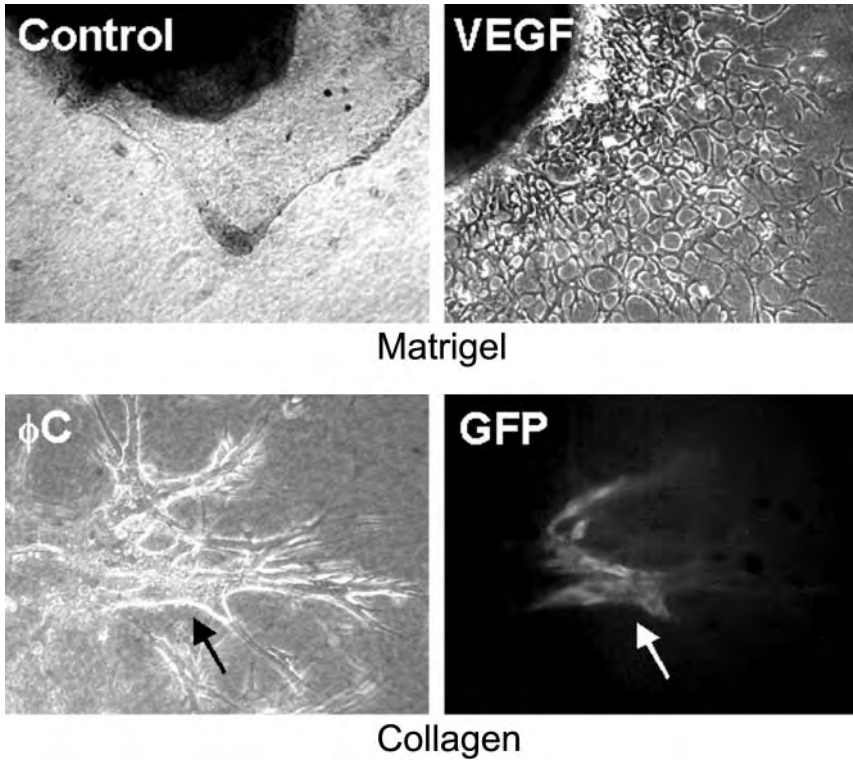

Fig. 3. Growing embryoid bodies (EBs) in matrix protein gels. EBs may be grown in either Matrigel or collagen gels, in which they retain their three-dimensional structures. Addition of angiogenic growth factors to either gel induces the outgrowth of green fluorescent protein $(GFP)^+$ ECs from the EBs. Note the formation of an extensive network in Matrigel supplemented with vascular endothelial growth factor (VEGF) (**top panel**). The **lower panel** shows that cells growing out of Tie-1-GFP EBs in a vascular endothelial growth factor-supplemented collagen gel are positive for GFP. ϕC, phase contrast microscopy.

stimulates the formation of a dense network of short vessels at the periphery of the EB, FGF-2 induces the formation of long and slender vessels originating at the center of the EB (*7* and our unpublished observations).

The development of vascular structures can also be monitored in real time, for example, as they grow out of EBs included in a gel of extracellular matrix proteins. **Figure 3** shows that GFP^+ vascular structure outgrowth is stimulated by angiogenic factors like VEGF directly added to the matrix protein gel.

Finally, differentiation of ECs in EBs provides a suitable model not only to understand the process of endothelial differentiation but also to identify molecules involved in the regulation of angiogenesis. For example, it has been used in our laboratory to characterize a VEGF-like factor isolated from vipera venom, a powerful pro-angiogenic factor *(9)*, and to identify histone deacetylases as potent angiogenesis inhibitors *(10)*.

1.2. Genetic Selection of ECs

Having access to large populations of ES cell-derived ECs is potentially very interesting, not only from a fundamental standpoint, but also because they might be used in the context of grafting or post-ischemic therapies, to improve the revascularization of damaged tissues (*see* **Subheading 1.3.**).

In 2000, Yamashita et al. *(11)* proposed a method to select endothelial progenitors by fluorescent-activated cell sorting on the basis of VEGFR-2 expression. Cells were also counterselected for E-cadherin expression, a marker of undifferentiated ES cells at this stage. However, VEGFR-2 is expressed at the very early stages of mouse ES cell differentiation and may even be expressed on undifferentiated monkey and human ES cells *(6,9)*, which in both cases increases the risk of selecting along unwanted undifferentiated cells. Moreover, VEGFR-2 is also expressed by a variety of non-ECs, including chondrocytes, smooth muscle cells (SMCs), and neuronal cells *(12–14)*.

An alternative strategy is genetic lineage selection based on the expression of an antibiotic resistance gene controlled by a "late" endothelial-specific promoter, namely, that of the orphan tyrosine-kinase receptor Tie-1 (this promoter was described in **refs.** *15* and *16*). As mentioned, Tie-1 is expressed later than VEGFR-2, and it is expressed only on ECs and their progenitors.

Recombinant ES cell clones were thus engineered that express a puromycin resistance gene under the control of the Tie-1 promoter. The same promoter was also used to drive the expression of the GFP in these cells to facilitate their detection both in vitro and in vivo. We have shown that the selection procedure gave rise to an endothelial population (pure at 96–98%; *see* **Fig. 4**) expressing a variety of endothelial markers and able to participate in blood vessel formation in vivo *(2)*.

During selection, ECs have a tendency to grow in very packed colonies (**Fig. 4**). A couple of days after puromycin removal, they adopt a morphology more characteristic of ECs in culture. **Figure 5** shows the morphology of an EC population that has been selected in puromycin-containing medium for 4 d and maintained in culture for an additional week without puromycin but in the presence of VEGF (**Fig. 5A**). The lower panels show that most cells expressing the early marker CD31 (B) have retained GFP expression, driven by the endothelial specific Tie-1 promoter (**Fig. 5B**).

The small percentage of non-ECs present in culture after selection were shown to be smooth muscle cells (SMCs) derived from ECs, as evidenced by the simultaneous expression of endothelial and SMC markers. Such a direct relationship between the endothelial and the SMC lineages was also observed by Yamashita and coworkers *(11)*, who suggested the existence of a common progenitor to both cell types. We have shown (**ref.** *2* and **Fig. 6**) that the emergence of SMCs in a previously selected endothelial population was strongly promoted by laminin-1 and transforming growth factor (TGF)-β, two known inducers of SMC differentiation *(17,18)*. Using similar ES cell differentiation models, studies have further highlighted the roles of VEGFR-2, the transcription factor Tal-1 *(19)*, and TGF-β *(20)* in regulating cell fate choice between the endothelial and smooth muscle lineages. These findings clearly showed that in vitro differentiation of ES cells may yield invaluable information on the mechanisms of cell differentiation.

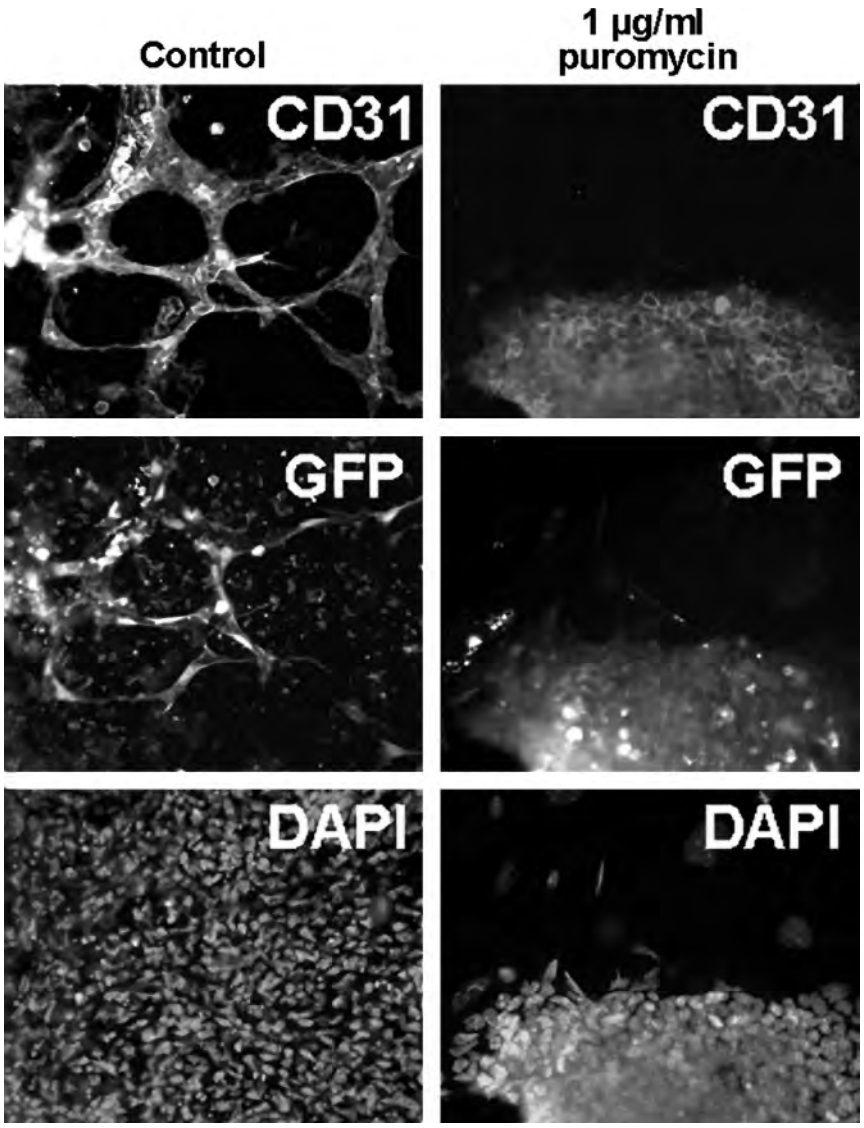

Fig. 4. Genetic selection of endothelial cells (ECs) from a heterogeneous population of differentiating embryonic stem (ES) cells. Recombinant ES cells were generated that express both green fluorescent protein (GFP) and a puromycin resistance gene under the control of the endothelium-specific promoter of Tie-1. Cells were differentiated in two dimensions and in the presence of VEGF for 7 d and treated or not with 1 µg/mL puromycin for 4 d to eliminate non-ECs. In the control population (not treated by puromycin), ECs are visualized as $CD31^+/GFP^+$ vessel-like structures or more compact colonies among other cell types (DAPI staining revealed the presence of a large number of non-ECs). In contrast, the population treated with puromycin for 4 d is almost exclusively endothelial at 96–98%, as shown by the superposition of CD31, GFP, and DAPI staining. Note that selection resulted in the compaction of ECs.

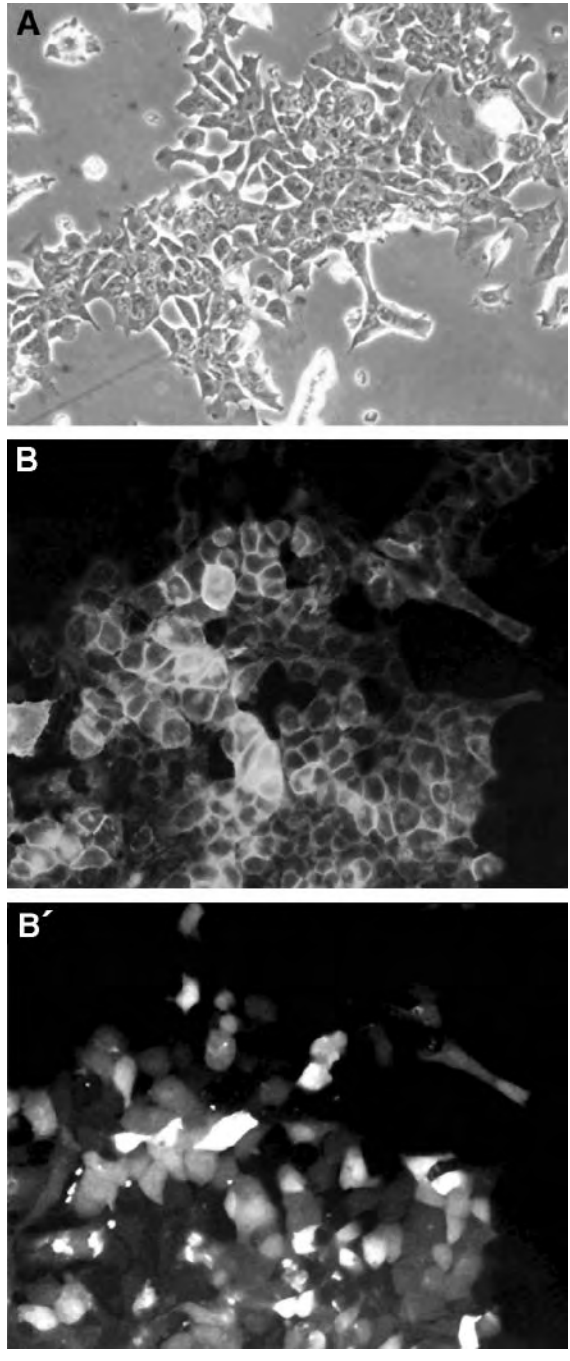

Fig. 5.

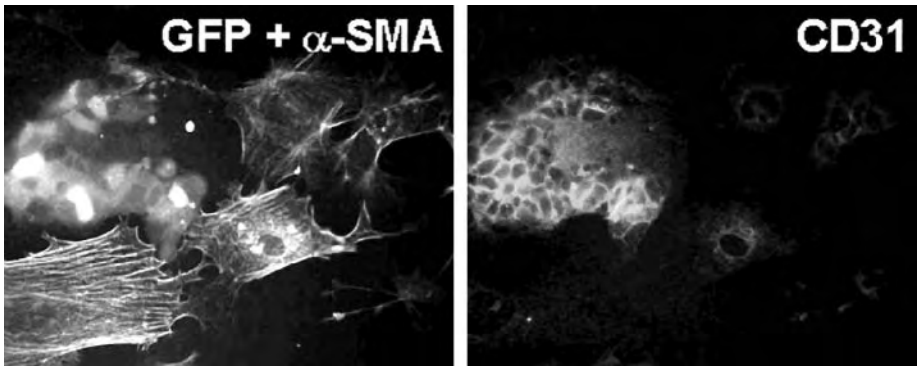

Fig. 6. Transforming growth factor (TGF)-β promotes the differentiation of endothelial cells (ECs) into smooth muscle cells (SMCs). Genetically selected ECs were treated for 48 h with 5 ng/mL TGF-β. ECs are positive for CD31 and for green fluorescent protein. SMCs are identified through the expression of α-smooth muscle actin, visualized as fluoresceine-labeled stress fibers in the large cells next to the endothelial colony.

1.3. Transplantation of ECs In Vivo

Transplantation of ECs resulting from genetic selection may be of major interest when working with the vascular system. Here are examples of issues they may help resolve:

1. If engineered to express fluorescent proteins, then they may be tracked down, pointing out the sites of intense neovascularization.
2. Combined with recombinant technology, EC transplantation may help define the role of specific proteins involved in the incorporation of circulating ECs into neovessels.
3. Transplanted ECs may be used in the future as biological "bricks" to reconstruct the vascular wall after injury or in postischemic conditions. Their ability to give rise to both components of the vascular wall, the EC, and the SMC could be especially valuable in this respect.
4. ECs might also be used as cellular vectors to deliver secreted factors, including pro- or antiangiogenic molecules, to the sites of neovascularization. This may not only increase our fundamental knowledge of the mechanisms underlying angiogenesis, but also be envisioned as a novel form of combined "transgenic/cellular" therapy.

Genetically selected ECs were tested for their ability to be incorporated in the neovasculature, using tumor angiogenesis in the nude mouse as a working model *(2)*. To this end, ECs were mixed with tumor cells (TCs) and injected in the nude mouse. **Figure 7** shows that tumors grown from this mixture (ECs + TCs) are much larger than tumors resulting from the injection of TCs alone, suggesting that tumor growth increased as a consequence of EC presence, possibly as a consequence of increased angiogenesis.

Fig. 5. *(Opposite page)* Morphology and endothelial marker expression of a genetically selected endothelial population. **(A)** Phase contrast microscopy of morphology of endothelial cells (ECs) 1 wk after selection. (**B**) and (**B′**) At 1 wk after selection, most cells that express CD31 (**B**) also express GFP (**B′**).

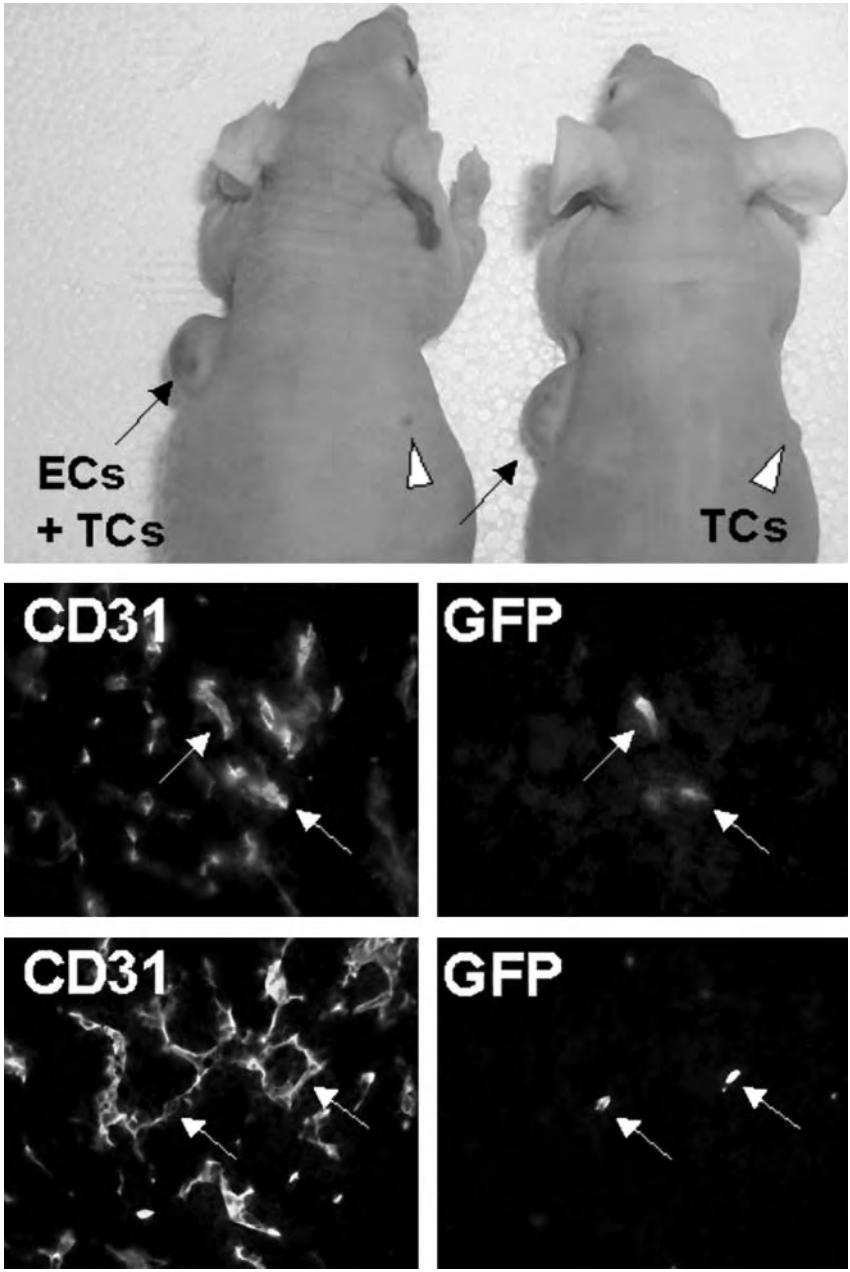

Fig. 7. Genetically selected endothelial cells (ECs) can participate to neovascularization in vivo. (**A**) Nude mice were injected with a mixture of selected ECs and tumor cells (TCs) (on the left flank) or with TCs alone (right flank). Note that the presence of ECs strongly promoted the growth of tumors. (**B**), (**B′**), (**C**), and (**C′**): green fluorescent protein$^+$ cells were found in the microvasculature of the tumors resulting from the mixture of ECs and TCs.

Injected GFP$^+$ cells incorporated into tumor blood vessels but, maybe surprisingly, were not found in large numbers (*see* **Fig. 7**).

Data presented by Marchetti et al. ***(2)*** also showed that, in tumors resulting from the injection of ECs with TCs, all blood vessels were covered by SMCs. In contrast, SMCs were almost absent in vessels of control tumors. It is possible that the microenvironment provided by this specific tumor stimulates the transdifferentiation of ECs into GFP $^-$ SMCs, which could explain the small number of GFP$^+$ cells in blood vessels (the Tie-1 promoter is not active in SMCs).

Yurugi-Kobayashi et al. ***(21)*** showed that ES cell-derived ECs selected by fluorescent-activated cell sorting on the basis of VE-cadherin expression could also be incorporated in tumor vascularization, thereby increasing blood flow. Interestingly, VE-cadherin$^+$ cells integrated blood vessels more efficiently than VE-cadherin$^-$ endothelial progenitors. Injected cells gave rise to both ECs and SMCs, the latter in much lower numbers. The efficiency of EC incorporation seemed to be higher than in our model, which may be related to the nature of the tumor used in their study, the C6 glioma. Indeed, this tumor is highly angiogenic and may be more permissive for EC rather than SMC growth. Alternatively, the protocol of EC injection might play an important role in the efficiency of EC incorporation. Hence, Yurugi-Kobayashi et al. ***(21)*** injected ECs in several points around tumors that had already reached a certain size instead of mixing both cell types prior to injection.

Importantly, our studies showed that, even when injected in high numbers, ECs genetically selected from ES cells did not give rise to teratomas. However, injecting a very large number of ECs (10^7) produced hemangiomas, which are blood-filled tumors of endothelial origin ***(2)***.

Further studies are necessary to optimize the conditions for EC transplantation, and it may be interesting, for example, to test their ability to be recruited at sites of neovascularization after injection in the systemic blood circulation. According to Yurugi-Kobayashi et al. ***(21)***, it is not a method of choice for ES cell-derived ECs. Finally, the work of Shen et al. ***(22)*** suggested that these cells could be used for endothelium lining in tissue-engineered blood vessels.

We describe protocols here for culture conditions promoting endothelial differentiation in both two-dimensional cultures and three-dimensional EBs. We also provide protocols for establishing recombinant ES cell clones. The last section is devoted to the injection of ES-derived ECs in vivo in a tumor model in the nude mouse.

2. Materials

2.1. Tissue Culture

1. ES cell line: for our studies, we use OLA 129 ES cells, a kind gift of Dr. A. Smith (Centre for Genome Research, Edinburgh, UK).
2. 0.1% gelatin solution: dissolve 1 g gelatin (Sigma, St. Louis, MO; cat. no. G-1890) in 1 L distilled water (dH_2O). The solution should be autoclaved.
3. 1X phosphate-buffered saline (PBS): 11.5 g Na_2HPO_4, 2 g KH_2PO_4, 80 ng NaCl, and 2 g KCl in 1 L dH_2O. The solution should be autoclaved.

4. 100 mL 0.5% trypsin-5.3 m*M* ethylenediaminetetraacetic acid (EDTA·4Na (Gibco Invitrogen, Breda, The Netherlands; cat. no. 15400-054): this is considered 10X trypsin-EDTA and should be diluted in PBS for obtaining either 1X trypsin-EDTA or 2X trypsin-EDTA.
5. 500 mL Dulbecco's modified Eagle's medium (DMEM) 1X, containing 4.5 g/L glucose, Glutamax I, and pyruvate (Gibco Invitrogen, cat. no. 31966-021).
6. 500 mL fetal bovine serum (FBS) fetal clone III (HyClone Laboratories, Logan, UT; cat. no. SH30109.03).
7. 100 mL 100X nonessential amino acids (Gibco Invitrogen, cat. no. 11140-035).
8. 100 mL penicillin-streptomycin (Gibco Invitrogen, cat. no. 15140-122).
9. Leukemia inhibitory factor (LIF) (Sigma, cat. no. L-5158): LIF from Sigma is 1000X concentrated; it should therefore be diluted 1000-fold in culture medium.
10 100 mL β-mercaptoethanol (β-ME) (Sigma, cat. no. M-7522): a stock solution of β-ME (10^{-1} *M*; 1000X) is prepared with deionized water in a 15-mL Falcon tube, and the tube is wrapped in aluminum foil. The 10^{-1} *M* solution of β-ME can be stored 3 wk at 4°C. Final concentration of β-ME in the culture medium is 10^{-4} *M*.
11. VEGF (Sigma, cat. no. V3513).
12. TGF-β (Sigma, cat. no. T7039).
13. Laminin-1 isolated from Engelbreth-Holm-Swarm tumors (Sigma, cat. no. L2020).
14. Matrigel basement membrane matrix (BD Biosciences, Bedford, MA; cat. no. 356237).
15. G418 (Gibco Invitrogen, cat. no. 066-1811).
16. Puromycin (Sigma, cat. no. P8833).
17. 50 mg/mL hygromycin B in PBS (Gibco Invitrogen, cat. no. 10687-010).
18. Lysis buffer for DNA preparation: 100 m*M* Tris-HCl pH 8.0, 5 m*M* EDTA, 0.2% sodium dodecyl sulfate, 200 m*M* NaCl. 12.1 g Tris-HCl, 1.86 g EDTA, 2 g sodium dodecyl sulfate, and 11.7 g NaCl in 1 L dH_2O. Adjust to pH 8.0. Proteinase K (10 mg/mL diluted in distilled water; Sigma, cat. no. P2308) should be added to lysis buffer right before lysing cells at a concentration of 100 µg/mL (dilute 100X in lysis buffer).
19. 100-mm tissue culture dishes (Falcon/BD Biosciences, cat. no. 353003; *see* **Note 1**).
20. Falcon Multiwell™ six-well tissue culture plates (Falcon/BD Biosciences, cat. no. 353046).
21. Falcon Multiwell 12-well tissue culture plates (Falcon/BD Biosciences, cat. no. 353043).
22. 96-well flat-bottom plates (Nunc, Roskilde, Denmark; cat. no. 167008).
23. 96-well round-bottom plates (Nunc, cat. no. 055245).
24. Glass cover slips, 18-mm diameter (Marienfeld, Bad Mergentheim, Germany; cat. no. 0111580).
25. 100-mm Petri dishes (VWR International/MERCK, Fontenay sous Bois, France; cat. no. T90HE).
26. Cryotube vials (Nunc, cat. no. 343958).
27. CappAero multichannel (eight-channel) pipets (5 50-µL and 25 200-µL; Lomb Scientific, Taren Point, Australia; cat. no. 50-8AZ and 200-8AZ, respectively).
28. Coulter counter (Beckman Coulter, Fullerton, CA; model Z1).
29. Electroporation cuvets and electroporator (Eurogentec, Seraing, Belgium).

2.1.1. Media

Complete medium: add 55 mL FBS (fetal clone III), 5 mL nonessential amino acids, and 0.5 mL penicillin-streptomycin to a 500-mL bottle of DMEM. LIF and β-ME are usually added directly to the culture dishes (10 µL each per 10-mL culture; *see* **Note 2**).

2.2. Immunofluorescence

1. 1X phosphate-buffered saline (*see* **Subheading 2.1., item 2**).
2. Paraformaldehyde in PBS (*see* **Note 3**): for 1 L, in a 2-L bottle, dilute 100 g paraformaldehyde in 1 L PBS. Heat at 70°C for 2 h with the cap of the bottle closed. Add 270 µL 8 *N* NaOH. Filter and check that the pH is equal to 7.4.
3. Triton X-100 (USB, Cleveland, OH; cat. no. 22686) in PBS.
4. FBS (any brand; used only for blocking).
5. Rat monoclonal anti-mouse CD31 antibody (clone MEC13.3; 0.5 mg/mL; BD Biosciences, cat. no. 553370).
6. Rat monoclonal anti-mouse CD34 antibody (clone RAM34; 0.5 mg/mL; BD Biosciences, cat. no. 553731).
7. Rat monoclonal anti-mouse VE-cadherin antibody (clone 75; 0.5 mg/mL; BD Biosciences, cat. no. 28091D).
8. Rat anti-mouse VEGFR2 antibody (clone Avas12a1; eBiosciences, San Diego, CA; cat. no. 14-5821).
9. Mouse monoclonal anti-α-smooth muscle actin antibody (clone 1A4; Sigma, cat. no. A2547).
10. Biotin-conjugated donkey anti-rat immunoglobulin (Ig) antibody (Jackson Immuno Research Laboratories, Westgrove, PA; cat. no. 712-065-153).
11. Alexa Fluor 594 donkey anti-mouse Ig antibody (red fluorochrome; 2 mg/mL; Molecular Probes, Eugene, OR; cat. no. A21203).
12. Alexa Fluor 488 donkey anti-mouse Ig antibody (green fluorochrome; 2 mg/mL; Molecular Probes, cat. no. A21202).
13. Alexa Fluor 594-coupled streptavidin (red fluorochrome; 80 µg/mL; Molecular Probes, cat. no. A24927).
14. DAPI (4′,6-diaminido-2-phenyindole; dilactate, Sigma, cat. no. D-9564).
15. Microslides, 75 × 25 mm (ESCO/Electron Microscopy Sciences, Hatfield; PA; cat. no. 2950R).
16. 100 mL mounting solution (Citifluor, Leicester, UK).
17. Transparent nail polish.
18. Leica DMR microscope (Leica Microsystèmes SAS, Rueil-Malmaison, France).

2.3. Reverse Transcription and Polymerase Chain Reaction

1. Trizol reagent (Gibco Invitrogen, cat. no. 15596-026).
2. Chloroform (VWR International/Merck, cat. no. 1.02445.1000).
3. Isopropanol (VWR International/Merck, cat. no. 1.09634.1000).
4. 75% ethanol (dilute ethanol from VWR International/Merck, cat. no. 1.11727.2500).
5. Omniscript Reverse Transcriptase (RT) Kit (Qiagen, Courtaboeuf, France; cat. no. 205111).
6. Oligo $(dT)_{12–18}$ (Gibco Invitrogen, cat. no. 18418-012).
7. RNase inhibitor (Gibco Invitrogen, cat. no. 15518-012).
8. Oligonucleotides: HPRT 5′-GCTGGTGAAAAGGACCTCC-3′ and 5′-CACAGGACTAGAACACCTGC-3′; Flk-1 5′-AGCTCTCCGTGGATCTGAAA-3′ and 5′-CCAAGAACTCCATGCCCTTA-3′; Flt-1 5′-CGGAAGCTCTGATGATGTGA-3′ and 5′-TATCTTCATGGAGGCCTTGG-3′; Tie-1 5′-CTCACTGCCCTCCTGACTGG-3′ and 5′-CGATGTACTTGGATATAGGC-3′; ICAM-2 5′-CCTGTCTCTTCTTATCCTGTTC-3′ and 5′-CACCCCGTAGGTGCCTGTCC-3′; h1 calponin 5′-TAACCGAGGTCCTGCCTACG-3′ and 5′-TGTGGGTGGGCTCACTCAGC-3′; SM22 5′-ATCAAGCTTCGCTACTCTCCTTCCAGTCCACAAACGACCA-3′ and 5′-ATCGGATCCCTTCCCTTTCTAACTGATGATCT G-3′.
9. *Taq* polymerase chain reaction (PCR) master mix kit (Qiagen, cat. no. 201443).
10. Agarose gel apparatus and reagents.

2.4. Injection of ECs In Vivo

1. Athymic nude mice (Harlan, Gannat, France).
2. 500 mL DMEM/25 m*M* HEPES medium (Gibco Invitrogen, cat. no. 42430-025) containing 0.1% bovine serum albumin, filtrated.
3. Angiogenic TC line and corresponding culture reagents: for example, the Ras-transformed CCL39 cells described in **Subheading 3.4.** were cultured in DMEM without phenol red containing 7.5% fetal calf serum (FCS) (Dutscher, Brumath, France), penicillin (50 U/mL), streptomycin sulfate (50 mg/mL), and G418 (400 µg/mL) (all antibiotics were from Gibco Invitrogen).
4. 1-mL syringes (Terumo, Guyancourt, France; cat. no. BS-01T).
5. 21-gage 2-in. needles (Terumo, cat. no. NN21-50R).

3. Methods

3.1. Tissue Culture

3.1.1. ES Cell Culture

ES cell colonies have a characteristic morphology (*see* **Fig. 1**). They are constituted of tightly packed cells with no visible individual cell boundaries. Cell individuation and spreading is a sign of cell differentiation and should be avoided. Such a differentiated phenotype usually means that cells have been kept in culture for an excessive number of passages (*see* **Note 4**).

When using ES cells for producing transgenic mice, it is advised to grow ES cells on a feeder layer of embryonic fibroblasts to prevent differentiation (with the exclusion of the clone selection procedure unless antibiotic-resistant fibroblasts are used). However, because our work involves several steps of ES cell clone selection, we chose to culture ES cells directly on gelatin in the presence of LIF. ES cells grown in these conditions are able to give many different types of cells in appropriate differentiation protocols, including endothelial, cardiac, and neuronal cells (*see* **Note 5**).

1. Coat as many wells of a six-well plate as there are cryotubes to be thawed with 0.1% gelatin for 30 min at room temperature.
2. Quickly thaw ES cell-containing cryotubes at 37°C and transfer the content (1 mL) of each cryotube into 10 mL ES cell culture medium (*see* **Note 6**). Centrifuge cells for 5 min at 200*g*.
3. Discard medium, resuspend cells in 3 mL complete culture medium plus LIF and β-ME and plate cells in one gelatin-coated well. Culture cells at 37°C in 5% CO_2.
4. Change medium the next day.
5. For cell passaging, wash cells gently with PBS and trypsinize them in 1X trypsin-EDTA for 4–5 min at 37°C (*see* **Note 7**).
6. Resuspend cells by pipetting up and down four or five times and add complete medium plus LIF and β-ME to trypsin. Do not centrifuge the cells after trypsinization as serum contained in complete medium inactivates trypsin.
7. Reseed the cells 1:5 to 1:20, depending on how long they have to be kept before the next passage (*see* **Note 8**).

3.1.2. Freezing

ES cells can be frozen in cold complete medium containing 10% dimethyl sulfoxide (DMSO).

1. Wash cells with PBS, trypsinize, and resuspend them as in **Subheading 3.1.1.**, **steps 5** and **6**.
2. Centrifuge for 5 min at 200*g* and resuspend in cold complete medium containing 10% DMSO. Typically, five 1-mL cryovials are frozen from a 100-mm dish and two cryovials from a well of a six-well plate.

3.1.3. Producing Recombinant ES Cells

This section gives a protocol for selecting ES cell recombinant clones, for example, cells carrying transgenes coding for GFP and for a puromycine resistance gene, under the control of the endothelium-specific promoter Tie-1, as they are described in **ref. *2***. These transgenes facilitate the detection of ECs and allow their selection, respectively.

3.1.3.1. Electroporation (*see* **Note 9**)

1. For each type of transfection, trypsinize cells that have been grown in a 100-mm dish to 80% confluency. Meanwhile, coat two 100-mm dishes with 0.1% gelatin (*see* **Note 10**).
2. Resuspend cells in complete medium, count them in a Coulter counter (*see* **Note 11**), and centrifuge them for 5 min at 200*g*.
3. Resuspend the cell pellet in 300 µL PBS.
4. Mix cells with 20–30 µg plasmidic DNA containing the gene of interest, for example, that of a GFP protein under the control of an endothelial-specific promoter. A plasmid encoding for a selectable marker (generally an antibiotic resistance gene) and controlled by a promoter active in ES cells should be co-transfected to allow recombinant clone selection (*see* **Note 12**). We generally use a quantity of selection plasmid five times smaller (i.e., 4–6 µg) than that of the plasmid coding for the gene of interest.
5. Adjust the volume of the mixture to 500 µL with PBS and transfer it to an electroporation cuvet. Electroporate cells with a gene pulser set at 500 V and 40 µF (*see* **Note 13**).
6. Let cells recover in the cuvet for 5 min at room temperature.
7. Resuspend cells in 20 mL complete medium with LIF and β-ME and seed them in two gelatin-coated 100-mm dishes. Electroporation gives a high rate of cell mortality, and the cell mixture may be sticky after the electric pulse. However, most surviving cells will generally contain plasmidic DNA.
8. Incubate cells overnight at 37°C and 5% CO_2.
9. Change medium the next day.
10. At 48 h after electroporation, refeed cells with complete medium with LIF and β-ME plus the appropriate drug. We use the following drug concentrations: G418 (when a plasmid coding for a neo^r transgene is used) at 200–400 µg/mL and Hygromycin B (when a plasmid coding for a hyg^r transgene is used) at 150–200 µg/mL. Two different concentrations may be tested if electroporated cells were seeded in two 100-mm culture dishes in **step 7**.
11. Every 2–3 d, rinse dishes with PBS and refeed with selection medium plus LIF and β-ME.
12. Individual colonies can be seen after 7–10 d.

3.1.3.2. Selecting and Culturing ES Cell Clones

After 10–12 d, clones can be scraped off the dish and cultured in 96-well plates. It is convenient to scrape the clones under the microscope (*see* **Note 14**). It is recommended to pick colonies displaying the typical phenotype of ES cells. Neglect clones that show signs of differentiation.

1. Coat 96-well plates with gelatin. The number of wells to be coated depends on the number of clones to be picked. Picking 30 clones is often enough when screening for additional transgenesis (*see* **Note 15**).
2. Prepare another 96-well plate, this one with a round bottom (*see* **Note 16**).
3. Wash cells in 100-mm dishes once with PBS and add 10 mL PBS to the dish.
4. Set the micropipet on 20 µL, push down the piston, and hold it down while scraping the colony. Aspirate cells and transfer them in one well of the round-bottom plate. Repeat the operation for every colony to be picked (*see* **Note 17**).
5. When all clones have been picked, pipet 20 µL 2X trypsin-EDTA in the wells using the multichannel pipet. Pipet up and down twice to homogenize.
6. Incubate 5 min at 37°C.
7. Pipet trypsinized cells up and down five times. Add 160 µL complete medium with LIF and β-ME to each well using a multichannel pipet. The final volume of each well should be 200 µL. This plate is called plate 1.
8. Culture 2–3 d at 37°C and 5% CO_2.
9. When cells are 60–70% confluent (*see* **Note 18**), passage plate 1 in two different 96-well plates (always use gelatin-coated plates). One will be used to freeze the clones (plate 2), the other one to isolate genomic DNA for PCR screening (plate 3). Fill those two plates with 100 µL complete medium plus LIF and β-ME per well. To split plate 1, aspirate and discard exhausted medium, rinse once with 200 µL PBS, incubate 5 min with 20 µL 1X trypsin-EDTA, and resuspend with 180 µL medium (the medium should contain antibiotic to maintain selection pressure; antibiotic concentration can be lowered by a factor 2, however). The final volume of 200 µL is then split in two, 100 µL for plate 2, 100 µL for plate 3.
10. Incubate 2 d (or to semiconfluency) before freezing or lysing cells for genomic DNA preparation.
11. Freeze by trypsinizing clones from plate 2 as indicated in **step 9**; stop trypsinization by adding complete medium containing 10% DMSO.
12. Homogenize by pipetting up and down with the multichannel.
13. Wrap Parafilm first around the plate to maintain the lid closed and wrap several layers of tissue paper around the plate. Use tape to maintain the paper layers tight.
14. Place for 2 h at –20°C before transferring to –80°C. Clones can be stored at –80°C while screening genomic DNA for recombinants (*see* **Note 19**).

3.1.3.3. Preparing Genomic DNA

1. Rinse cells in plate 3 twice with PBS.
2. Add 150 µL lysis buffer containing 100 µg/mL proteinase K, transfer to microcentrifuge tubes, and incubate at 50°C for at least 2 h.
3. Add 150 µL isopropanol to each tube.
4. Mix by shaking the tubes vigorously but do not vortex to avoid breaking genomic DNA.
5. Centrifuge tubes 5 min at 16,000*g*.
6. Discard supernatant and wash once in 75% ethanol.
7. Centrifuge tubes 5 min at 16,000*g*.
8. Discard supernatant and let the pellets dry either in laminar flow or with a speed-vacuum device.
9. Resuspend pellets in 100 µL H_2O for at least 1 h at 50°C. Do not vortex.

3.1.3.4. PCR Screening

PCR screening allows the identification of those clones that have stably integrated the transgene of interest into their genome. In our example, this will be either the GFP

transgene or the puro^r transgene (ES clones carrying both transgenes were established sequentially; *see* **ref. 2**).

1. For each genomic DNA sample, mix 12 µL master mix, 1 µL of each oligonucleotide (from a 10 µ*M* stock), 10 µL sterile water, and 1 µL DNA (*see* **Note 20**).
2. Negative controls (one containing no DNA plus one containing DNA extracted from wild-type ES cells) and a positive control (10 ng plasmidic DNA; in our example, the plasmid coding for GFP or for puro^r) should be included.
3. Run PCR. Conditions depend on the oligonucleotide Tm and on the length of the DNA fragment to be amplified.
4. Run PCR products on 1–2% agarose gels depending on the size of the amplified product.

3.1.3.5. Thawing Recombinant Cell Clones

1. Gelatin coat wells from a 12-well plate, with the number of wells corresponding to the number of clones to be thawed.
2. To thaw selected clones, place the frozen 96-well plate at 37°C. As soon as one "recombinant well" starts to thaw, add some warm medium to it to accelerate the process.
3. Transfer the content of the thawed well into 1 mL complete medium with LIF and β-ME in a gelatin-coated well from a 12-well plate.
4. Incubate at 37°C.

3.1.4. Differentiation of ES Cells Toward the Endothelial Lineage

We use two different protocols for inducing the differentiation of ES cells in ECs. The first protocol involves EB formation; these are three-dimensional structures. In the second one, cells are differentiated in two dimensions. For differentiation protocols, complete ES cell medium with β-ME but without LIF should be used.

3.1.4.1. EB Formation

1. Trypsinize the ES cells as in **Subheading 3.1.1.**, **step 5**.
2. Resuspend cells in complete medium without LIF but with β-ME and count them.
3. Make a cell suspension containing 4.10^4 cells/mL using complete medium with β-ME but without LIF. To induce EC differentiation in EBs, 10 ng/mL VEGF or other angiogenic factors may be added to the differentiation medium. Angiogenic factors should be maintained along differentiation (i.e., added to the medium every other day).
4. Deposit 20-µL drops (each drop contains 800 cells) on the lids of bacteriological 100-mm Petri dishes (*see* **Note 21**). More than 50 drops can be made per dish.
5. Return the lid to cover PBS-filled dishes and incubate cells at 37°C and 5% CO_2 (*see* **Fig. 1**). This is d 0 (D0) of differentiation (*see* **Note 22**).
6. Observe EBs on d 3 (D3; *see* **Note 23**). To do so, lids are first transferred from the PBS-filled dish to an empty dish. The latter dish is then reverted upside down to allow observation of EBs with inverted objectives. Drops hold firmly enough to bacteriological Petri dishes and normally stay round in the reverting process.
7. EBs can be grown in hanging drops for up to 2 wk, possibly longer, as long as medium is refreshed two or three times a week. To change medium of hanging drops, revert the dish lid, incline the lid sufficiently (approx 45°) to allow EBs to "fall" at the bottom of the drop and pipet 15 µL medium from the top of the drop. The EB should remain on the lid. Then, pipet down 20 µL fresh medium onto the same EB (the difference in volume accounts for evaporation). Do not let the EBs dry.

8. From D3 or D4, EBs can also be plated into gelatin-coated wells or on gelatin-coated cover slips if immunofluorescence is to be performed (*see* **Subheading 3.2.** and **Note 24**). Fill the wells of 12-well plates with 1 mL complete medium with VEGF and β-ME but without LIF (*see* **Note 25**). Set the Pipetman to 20 µL, aspirate each EB-containing drop, and deposit the EB into the well (*see* **Note 26**). EBs can be used at any stage of differentiation for immunofluorescence, RT-PCR (*see* **Subheading 3.3.**), and Western blot analysis (*see* **Note 27**).

Figure 1 shows that vessels start to form around D6 or D7 and are fully developed around D10 or D12. Growing EBs for a longer time does not necessarily give better results and increases cell death. The protocol for immunostaining is given in **Subheading 3.2.**

3.1.4.2. Inclusion of EBs in Matrix Protein Gel

Differentiated EBs can also be included in a gel of type I collagen or a gel of basement membrane proteins (Matrigel). In such gels, EBs retain their full three-dimensional structure. Addition of angiogenic factors to the gel or to the culture medium above the gel will result in the outgrowth of endothelial pseudovascular structures. ES cells expressing GFP under the control of an endothelium-specific promoter are especially valuable in this case as it is difficult for antibodies to reach cells in polymerized gels.

1. For collagen gels, prepare a 2 mg/mL type I collagen solution by diluting the collagen stock solution (4 mg/mL) in EB culture medium. To do so, mix 100 µL stock solution with 100 µL medium in one well of a 12-well plate. For Matrigel, thaw a sample of Matrigel on ice and pipet 200 µL in one well of a 12-well plate.
2. Incubate either gel for 45 min at 37°C in a humidified incubator to allow polymerization.
3. Place EBs on top of polymerized collagen or Matrigel and cover with culture medium supplemented with 10 ng/mL VEGF.

Figure 3 shows that VEGF induces the specific outgrowth of GFP^+ ECs in both Matrigel and collagen gels. Outgrowth of GFP^+ cells can be followed day after day if the culture microscope is equipped with an ultraviolet lamp (lower panel of **Fig. 3**).

3.1.4.3. Endothelial Differentiation in Two-Dimensional Cultures and Genetic Selection

ES cells can also give rise to cells of the endothelial lineage when grown in two dimensions in the presence of angiogenic factors. This protocol is especially suitable for genetically selecting ECs from a heterogeneous population of differentiated ES cells (*see* **Note 28**).

1. Trypsinize ES cells as indicated for routine culture.
2. Resuspend cells in complete medium with β-ME and without LIF and count them.
3. Seed the cells at a density of 3×10^3 cells/cm^2 in either 100-mm tissue culture dishes (approx 60 cm^2) or in wells from a six-well culture plate (approx 10 cm^2). Add 10 ng/mL VEGF to the medium.
4. Incubate the cells for at least 4–5 d before changing medium. However, 10 ng/mL VEGF should be added every other day to the medium to promote EC differentiation and proliferation. Cells can be transferred to cover slip-containing wells at any stage of the culture if immunofluorescence is to be performed. In this case, cover slips should be coated with gelatin.

3.1.5. Differentiation of ECs Into SMCs

The differentiation of ES-derived ECs into SMCs can be promoted by growing cells in the presence of either TGF-β or laminin-1, provided as a coating.

1. Differentiate cells in two-dimensional cultures (as described in **Subheading 3.1.4.3.**) up to D9 or D10.
2. Trypsinize and grow cells either on gelatin in the presence of 5 ng/mL TGF-β or on laminin-1-coated plates (*see* **Note 29**).
3. After 24–48 h, observe cells by phase contrast microscopy. Although ECs exhibit a cobblestone phenotype with numerous intercellular junctions, SMCs can be identified through morphological changes toward a more elongated, fibroblastlike phenotype.
4. Expression of SMC markers can be monitored by immunofluorescence (*see* **Subheading 3.2.**) or RT-PCR (*see* **Subheading 3.3.**).

Figure 6 shows that TGF-β promotes the emergence of large cells positive for α-smooth muscle actin and negative for CD31, next to a colony of $GFP^+/CD31^+$ ECs. Note that the α-smooth muscle actin signal organized in bundles (which are actin cables) is clearly absent in ECs, which only show a diffuse GFP signal. We have also shown by RT-PCR that SMCs present in these cultures express two SMC markers, SM22 and h1 calponin (our unpublished results).

3.2. Immunofluorescence

Both ECs or whole EBs can be analyzed by immunofluorescence for the expression of endothelial markers (*see* **Note 30**).

1. Wash cells or EBs with PBS in culture plates.
2. Fix cells or EBs in 3% paraformaldehyde/PBS for 15 min at room temperature (*see* **Note 31**). Wash cells three times with PBS.
3. Permeabilize the cells with 0.2% Triton X-100/PBS for 3–5 min at room temperature. This step permeabilizes plasma membranes and allows antibody entry inside the cells and inside EBs.
4. Wash cells three times with PBS.
5. Incubate cells with 20% FBS/PBS for 1 h at room temperature to prevent binding of antibodies to unspecific binding sites.
6. At this point, it is best to take cover slips out of the plates and put them upside up on Parafilm (*see* **Note 32**). Write, directly on the Parafilm, the culture conditions and the antibody used for each cover slip.
7. Dilute antibodies in 20% FBS/PBS. The following dilutions were used for endothelial differentiation: 1/1000 anti-CD31; 1/1000 anti-CD34; 1/500 anti-VE-cadherin; 1/500 anti-VEGFR2; and 1/1000 anti-smooth muscle actin.
8. Incubate each cover slip with 100 µL of the appropriate antibody dilution for 60–90 min at room temperature. Use a box lid to cover the cover slips.
9. Wash cover slips with PBS. For cells grown in two dimensions, washing efficiency is increased by pipetting down PBS on one side and removing it simultaneously on the other side with an aspiration pump. This procedure creates a PBS flux that allows rapid and efficient washing. However, this should not be used for washing EBs as they sometimes poorly adhere to cover slips. In this last case, PBS should be pipetted down on all cover slips and, in a second step, should be removed by aspiration. Repeat at least three times and let the PBS stand for 5 min the last time.

10. Incubate cover slips with secondary antibodies coupled either to fluorochrome or to biotin for 60 min at room temperature. The following dilutions are used in our lab: 1/500 biotin-conjugated antirat Ig antibody and 1/250 Alexa-coupled antimouse Ig antibodies. Optionally, cell nuclei can be visualized by adding DAPI to the secondary antibody dilution. This gives an estimation of the percentage of cells positive for a given marker.
11. Wash cover slips as in **step 9**.
12. If biotin-coupled antibodies were used, then cover slips should be incubated with fluorochrome-coupled streptavidin for 30–60 min at room temperature, protecting cover slips from light.
13. Wash cover slips as in **step 9**.
14. To prepare microslides, write on the left side a code number or the name of the antibody and deposit two drops (approx 20 µL) of Cityfluor mounting medium (or any other mounting medium) on the slides, with sufficient space in between to allow two cover slips to be mounted on each slide.
15. Detach the cover slip from the Parafilm with tweezers, drain remaining liquid by holding the edge of the cover slip against tissue paper, and revert the cover slip upside down on the Cityfluor drop (*see* **Note 33**).
16. Seal the cover slips with nail polish and let dry before observing with a fluorescence microscope (*see* **Note 34**).

3.3. Reverse Transcriptase Polymerase Chain Reaction

3.3.1. Trizol RNA Extraction and Denaturation

The protocol for RNA extraction and denaturation is derived from the manufacturer's instructions (*see* **Note 35**).

1. Lyse cells by adding Trizol reagent, 1 mL/10 cm^2.
2. Incubate for 5 min at room temperature.
3. Add 0.2 mL chloroform per 1 mL Trizol. Cap tubes securely and shake vigorously by hand for 15 s.
4. Incubate 5 min at room temperature.
5. Centrifuge at 12,000*g* for 15 min at 4°C.
6. The mixture separates into two phases, a phenol/chloroform lower phase (red) and an upper aqueous phase (colorless), containing RNA.
7. Transfer the aqueous phase to a fresh tube and precipitate with isopropanol using 0.5 mL isopropanol for 1 mL Trizol reagent used in **step 1**.
8. Incubate for 10 min at room temperature and centrifuge at 12,000*g* at 4°C.
9. Discard supernatant and wash pellet with 1 mL 75% ethanol per 1 mL Trizol reagent used. Centrifuge at 7000*g* for 5 min at 4°C.
10. Air-dry the RNA pellet (*see* **Note 36**).
11. Resuspend RNA in sterile water. Incubate for 10 min at 55°C to help dissolve it.
12. Measure absorbance of diluted sample (*see* **Note 37**).
13. At this point, RNA can be either conserved at –80°C or denatured by incubating for 5 min at 65°C and set on ice next if RT is to be performed.

3.3.2. Reverse Transcription

For RNA quantification, RNA is transcribed into single-stranded complementary DNA using an Oligo-dT primer that specifically hybridizes to the poly-A tail of mRNAs. This protocol is derived from the manufacturer's instructions.

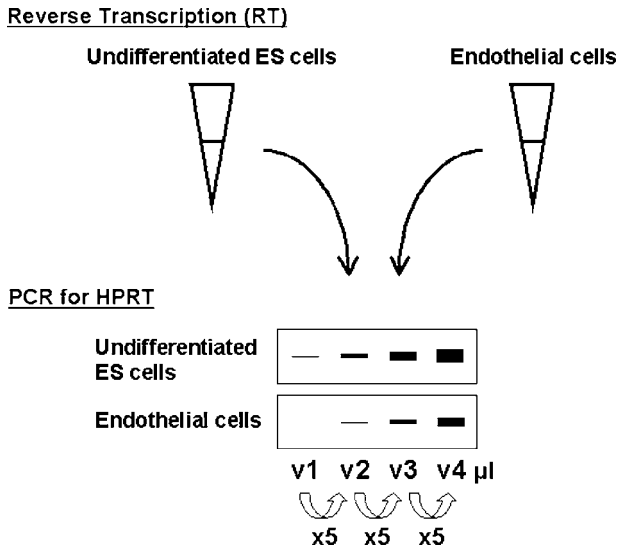

Fig. 8. Setting up reverse transcriptase polymerase chain reaction (RT-PCR) conditions. To allow adequate quantification of mRNA, the PCR following the RT step should be performed in unsaturated conditions and should include quantification of mRNAs coding for a housekeeping gene, such as hypoxanthine phosphoribosyltransferase (HPRT). The PCR for HPRT indicates the respective volumes of undifferentiated RT and endothelial RT to be used. In the present scheme, a volume v2 of the undifferentiated RT reaction gives the same amount of HPRT PCR product as a volume v3 (twice the size) of the endothelial cells RT reaction. *See* **Subheading 3.3.3.** and **Note 38** for further details.

1. Prepare RT mix: 10X buffer RT (2 µL); dNTP mix (2 µL; the mix contains 5 m*M* of each dNTP); Oligo-dT primer (2 µL); RNase inhibitor (1 µL); Omniscript RT (1 µL); template RNA (1 µg); and RNase-free water (QSP 20 µL).
2. Incubate for 60 min at 37°C and store on ice before proceeding with the PCR reaction.

3.3.3. Polymerase Chain Reaction

In the context of ES cell differentiation, RT-PCR is typically used to compare gene expression in undifferentiated and differentiated ECs. To estimate the respective amounts of "undifferentiated" and "endothelial" RT reactions to be used, we usually first compare the amounts of mRNA coding for a housekeeping gene, hypoxanthine phosphoribosyltransferase (HPRT), the levels of which generally do not vary (*see* **Fig. 8** and **Note 38**).

1. Proceed as in **Subheading 3.1.3.4.** using the *Taq* master mix from Qiagen, with the temperature of annealing depending again on the oligonucleotide sequences.
2. Run PCR products on 1–2% agarose gels, depending on the size of the amplified product.

3.4. In Vivo Properties of Genetically Selected ECs

We tested the ability of genetically selected ECs (*see* **Subheading 3.1.5.**) to be incorporated to newly formed blood vessels in a vascularized tumor model in the

Table 1
Protocol of Endothelial and Tumor Cell Injection

Type of tumor	Concentration	Injection
Tumor cells (TCs)	5.10^6 TCs/mL	200 µL (10^6 TCs)
Endothelial cells (ECs)	5.10^6 ECs/mL	200 µL (10^6 ECs)
	5.10^7 ECs/mL	200 µL (10^7 ECs)
TCs + ECs	5.10^6 TCs + 5.10^6 ECs/mL	200 µL (10^6 of each)

athymic nude mouse. ECs were mixed, prior to injection, with cells showing a low tumorigenic potential. The TC line we used in **ref.** *2* was of hamster origin and was transformed with an active form of MEK *(23)*, but many other tumor types may be tried.

1. Calculate the number of ECs and TCs needed for the experiment well ahead of the day of injection and grow cells accordingly. Mice are injected with a mixture of 10^6 ECs and 10^6 TCs on the left flank and with 10^6 TCs alone on the right flank. Additional animals were included for controls, for injection of 10^6 ECs alone, or for injection of 10^7 ECs alone (*see* **Table 1** and **Note 39**).
2. The day of injection, trypsinize ECs as indicated previously. Trypsinize TCs as for routine culturing. Wash cells once in their respective medium containing serum.
3. Resuspend each cell type in sterile DMEM/25 m*M* HEPES containing 0.1% bovine serum albumin (to prevent cells from sticking to each other) at the concentrations indicated in **Table 1** and place the tubes on ice.
4. Mix ECs and TCs when required (according to **Table 1**).
5. Inject animals with 200 µL of either cell mixture using a fresh needle for each mouse.
6. Let tumors grow for several weeks depending on the tumor growth capacity, but keep a record of this growth every few days (*see* **Note 40**).
7. Sacrifice animals when desired and process tumor tissues for paraffin embedding or freezing (*see* **Note 41**).

4. Notes

1. Other brands of tissue culture dishes can be used as well because the dishes are gelatinized before plating cells.
2. To remain in their undifferentiated state, ES cells must be cultured in the presence of LIF and β-ME. However, we do not add LIF and β-ME to the 500-mL bottle as LIF is labile and β-ME is light sensitive. Rather, we prepare a 100-mL bottle of medium, add both LIF and β-ME to it, and protect the bottle from light by aluminum foil. Alternatively, LIF and β-ME may be added to the medium directly in the culture dish.
3. Work under a laminar flow as paraformaldehyde is toxic.
4. Burdon et al. *(24)* showed that ERK signaling is not required for ES cell self-renewal, in contrast to other cell types. This means that an important recombinant ES cell clone that shows signs of differentiation may be "rescued" by the addition of a MEK inhibitor (such as PD098059 or U0126) in the culture medium. This will eliminate cells that are committed toward differentiation.
5. This procedure is for routine ES cell culturing. The specific procedure for isolating recombinant ES cell clones is given below in **Subheading 3.1.3.2.**
6. When thawing a new batch of ES cells (the freezing procedure is given in **Subheading 3.1.2.**), cells should be plated on six-well multidish plates to ensure sufficient confluency that helps cells to recover.

7. Split cells before confluency as confluency rapidly induces differentiation. The following volumes of PBS, trypsin-EDTA, and complete medium are used when passaging the following types of culture dishes:
   a. 100-mm dishes: 10 mL PBS for washing, 3 mL trypsin-EDTA, and 7 mL complete medium plus LIF and β-ME to stop the trypsinization and resuspend the cells.
   b. Six-well plates: 3 mL PBS, 0.5 mL trypsin-EDTA, and 2.5 mL complete medium plus LIF and β-ME.
8. When starting with ES cell culturing, one should plate several dilutions to optimize passage conditions, as growth may vary from one cell line to another.
9. When ES cells are used for the production of genetically modified animals, the method of choice for transfection is electroporation. Other transfection methods, such as calcium phosphate ($CaPO_4$) transfection may result in the formation of concatemers of linearized fragments of DNA; electroporation does not. Concatemer formation may be a problem when homologous recombination and gene replacement are to be achieved, for example, for knockout or knockin mice production. But, in other instances, ES cells can also be efficiently transfected by the calcium phosphate method. In any case, the general rule is to select several recombinant clones to avoid unspecific effects caused by the locus of transgene integration in the genome.
10. It is advised to include an electroporation condition with no plasmidic DNA. This will constitute a useful control during the drug selection step.
11. There should be approx $2–3 \times 10^7$ cells.
12. Promoters active in undifferentiated ES cells include the phosphoglycerokinase promoter and the pCAG promoter, a combination of chicken β-actin promoter and cytomegalovirus immediate-early enhancer (described in **ref. *25***).
13. Optimal conditions for electroporation must be established for each type of apparatus.
14. To do so, install the microscope in the tissue culture hood (the hood must have an electric window) and pick clones while looking through the microscope. This can be done relatively easily with practice. If clones are sufficiently apart from each other, then they may be picked without a microscope.
15. Picking several hundred clones is sometimes necessary to identify homologous recombination events.
16. It also works with flat-bottom plates.
17. Do not pipet PBS up and down when scraping the colony as it will scatter cells in the dish, increasing the risk of obtaining heterogenous clones.
18. It is actually quite rare that all clones reach subconfluency simultaneously. It is not a problem, and it is better to passage and even freeze wells in which cells are only 20% confluent than to let cells differentiate because of high confluency.
19. This may take a few days.
20. It is advised to make a mix for all clones to be tested with master mix, oligonucleotides, and sterile water and to distribute 23 µL in each PCR tube before adding 1 µL DNA to each.
21. Petri dishes are used to avoid EBs sticking to the plastic.
22. Special care should be taken not to knock the dishes against the bench, microscope, incubator, or the like. Also, the incubator's door should not be slammed to avoid drops falling into the PBS. This is really to be taken into consideration if other people use the same incubator (it is a good idea to put a sign on the door).
23. We usually do not observe EBs before d 2 (D2) to avoid disturbing the aggregation process. Later, EBs can be observed with a regular phase contrast microscope used for routine culture, with objectives usually inverted.
24. This is usually the method we use to study endothelial differentiation.

25. This medium may be complemented with specific differentiation factors. Supplementation with VEGF may also start at D3 instead of D0.
26. At this stage of growth, EBs can be seen as a small whitish dot in the yellow tip, which is convenient to follow them during the transfer procedure. It is rather difficult to leave EBs behind on the lid of the Petri dish as they can be readily seen as very small beads when liquid has been removed. If an EB does not come up with the first aspiration, then simply pipet the volume down again and try to aspirate it at the second try. Of course, extensive up-and-down pipetting should be avoided as it can damage the EB. EBs sometimes tend to be attracted by the periphery of the well, over the edge of the cover slip. Therefore, we usually deposit four or five EBs in each well for security. They may eventually fuse together or stay as single EBs on the bottom of the well.
27. Although the latter type of experiments requires large numbers of EBs, plan at least 15 EBs for one RT-PCR point.
28. Although it is theoretically possible to purify ECs from EBs, we have observed that enzymatic dissociation of EBs with trypsin or collagenase is associated with high cell mortality. Therefore, we favor EC differentiation in two dimensions for genetically selecting ECs. The concentration of antibiotic should be adapted to the strength of the promoter used and the beginning of the treatment adapted to the onset of the promoter activity during differentiation. When using the promoter Tie-1, we add puromycin to differentiated cells at D7 as the onset of its activity is around D5. Using the same promoter, 1–2 µg/mL puromycin should be used to select ECs and maintained in the medium for 4 d. Cell selection is accompanied by gradual decrease of the Tie-1 promoter activity, as visualized by downregulation of GFP expression. It is possible that the transcriptional activity of the Tie-1 promoter is regulated by other cell types present in the unselected culture. Because the transcription of $puro^r$ is also controlled by the Tie-1 promoter, it is therefore not recommended to maintain the selection for more than 4 d as it may eventually kill the cells. We prefer to start a round of differentiation/selection every time we need cells for a set of experiments rather than keep selected cells for a long time in culture as they may quickly lose the expression of the "late differentiation" markers. Nevertheless, Tie-1-expressing cells may be "reselected" approx every 2 wk with a 4-d treatment with 1 µg/mL puromycin. In any case, the maintenance of a "differentiated" state can be judged along the culture on the basis of GFP expression because it is controlled by the Tie-1 promoter. This can be checked on live cells as long as the microscope is equipped with a strong enough ultraviolet lamp. Finally, it should be noted that selected cells can be efficiently transfected using the $CaPO_4$ method.
29. For laminin-1 coating, prepare a solution of 20 µg/mL laminin-1, diluted in water, and incubate overnight at 4°C in cover slip-containing wells. Wash three times with PBS the next day before seeding the cells.
30. For analysis of marker expression in immunofluorescence, cells or EBs should be grown on 18-mm cover slips in 12-well plates for the appropriate number of days. Before coating with gelatin, cover slips should be sterilized either by dipping them in ethanol and flaming them or using a microwave, with the cover slips directly in the culture plates (twice for 2 min, at maximal power, with a beaker of water to prevent the plate from melting).
31. Paraformaldehyde is toxic and should be manipulated in a laminar flow hood.
32. This saves antibody as the volume of the antibody solution can be lowered to 80–100 µL, whereas 300–400 µL are needed to cover the cover slip in a well from a 12-well plate. Parafilm should be taped tightly on a plane surface (e.g., the bench) to avoid the formation of "waves." To take cover slips out of the plate, use tweezers and a needle with its extremity slightly bent. It is not mandatory to use a humid chamber to perform this immunofluorescence staining as it works very well on the bench unless the time of incubation with the antibody

solution is lengthened. In this case, cover slips may dry, which causes high background staining. However, it is always necessary to protect the cover slips from light, especially while incubating with fluorochrome-coupled antibodies.

33. Again, cover slips should be protected from light.
34. The edge of the cover slip, and not the center, should touch the Cityfluor drop first to avoid bubble formation. Gently press down the cover slip against the slide with the needle and remove the mounting liquid in excess by aspiration. EBs grown on cover slips have a certain three-dimensional volume, but they can be gently flattened between cover slip and slide. This will not have any effect on the morphology of pseudovascular structures, generally found at the flat periphery of the EB, at least in the case of experiments performed with VEGF.
35. One should wear gloves during the whole process of RNA preparation and work with disposable plasticware and sterile tubes.
36. Overdried RNA is difficult to resuspend.
37. The concentration of RNA sample is given by [C µg/mL] = $40 \times A_{260} \times$ Dilution factor.
38. If real-time RT-PCR is not available, then it is important to set up PCR conditions in such a way that the reaction is not performed in saturating conditions but in the exponential phase of PCR. If performed in saturating conditions, then variations in gene expression may be missed. As HPRT mRNAs are abundant, several dilutions of each RT reaction should be tested (up to 1/100 to 1/1000 of the RT when starting with 1 µg RNA). Comparing the "slopes" of HPRT amplification in undifferentiated and endothelial conditions allows estimation of the volumes of each RT to be used to adequately compare gene expression. On the schematic example shown on **Fig. 8**, v1, v2, v3, and v4 represent PCR reactions performed with increasing volumes of each RT. It shows that the same amplification for HPRT is obtained with a volume v2 for the undifferentiated RT and with a five times bigger volume, v3, for the endothelial RT. The same volume ratio should therefore be conserved when comparing mRNA amounts for endothelial markers. However, it should be kept in mind that the mRNAs coding for most proteins, including endothelial markers, are much less abundant than those of housekeeping genes. Thus, if the ratio should be conserved, then volumes should be upscaled. Typically, if RT must be diluted to 1/100 to 1/1000 for HPRT amplification, then 1–2 µL RT give a sufficient amplification for endothelial marker genes. For security, it is advised to make a small dilution curve for quantification of endothelial marker RNAs as well to check that one does not work in saturated conditions.
39. Experimentation on animals should follow the rules currently in effect in the country in which the work is conducted. As a general rule, the weight of the tumor should not exceed 10% of the weight of the mouse.
40. Controls are included for the following reasons: (1) to check for the absence of teratoma formation, which may arise from ES cells that might have escaped differentiation and selection in vitro; and (2) to assess the ability of genetically selected ECs to form endothelial tumors when injected in large number.
41. Some antibodies may not work well on paraffin-embedded sections, but frozen sections are generally of sufficient quality for the analysis of vascular density and the detection of GFP^+ cells. If frozen sections are to be used, then follow the procedure indicated in **Subheading 3.2.** for immunofluorescence.

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22

Integrins and Vascular Development in Differentiated Embryonic Stem Cells In Vitro

Sheila E. Francis

Summary

The development of techniques for the in vitro differentiation of mouse embryonic stem (ES) cells has provided researchers with the tools to investigate vascular development as would occur in an early embryo. Mice lacking the vascular integrins have various defects in the vasculature, including defects in vessel architecture and hemorrhage: some animals die before birth. Using homozygous null ES cells for these integrins and in vitro differentiation of vascular networks in embryoid bodies, we can recapitulate the defects that give rise to embryonic lethality in mice. This allows the investigation of the molecular and cellular mechanisms responsible for the vascular defects that arise in the embryo.

Key Words: Embryoid bodies; embryonic stem cells; endothelial cells; integrins.

1. Introduction

An intact circulation is essential for life. Initially, in early life, a primitive network of vessels develops by a process called vasculogenesis in which endothelial precursor cells proliferate, migrate, and merge. Later, vessels in this network form additional tubes by further growth, sprouting, and subdivision during a process known as angiogenesis *(1)*. Both vasculogenesis and angiogenesis are dependent on interactions between cells, their basement membranes, and surrounding extracellular matrix. These interactions are mediated by specialized cell adhesion receptors called integrins and other signaling molecules, such as platelet-derived growth factor (PDGF) or Notch.

The complement of integrins and their ligands found on vascular cells is well known *(2)*. These are $\alpha5\beta1$ and its ligand fibronectin, $\alpha1\beta1$ and $\alpha2\beta1$ and their ligands, the laminins and collagens, as well as $\alpha v\beta3$ and $\alpha v\beta5$ binding to vitronectin, fibronectin, and thrombospondin *(3,4)*. One important aim of investigators in the vascular integrin field has been to determine which of these integrin receptor-ligand systems are required mediators of vascular development.

This work was carried out on sabbatical in the laboratory of Richard O. Hynes, Center For Cancer Research, Massachusetts Institute of Technology, Cambridge, MA.

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The differentiation of mouse embryonic stem (ES) cells in vitro provides a useful model system in which to study the development of a number of cell lineages, including endothelium. Usually, totipotent ES cells proliferate and maintain an undifferentiated state indefinitely on a feeder layer with leukemia inhibitory factor (LIF). Once LIF is removed, cells begin to differentiate into simple or cystic embryoid bodies (EBs) *(5)*. These are collections of ES cells surrounded by ectodermal or endodermal cells, with a fluid-filled (cystic) cavity, similar to early embryos *(6)*.

We describe here protocols for the differentiation of ES cells toward an endothelial cell lineage using a three-dimensional methylcellulose-based cell culture system and analysis of these networks using confocal microscopy.

2. Materials

2.1. Tissue Culture

1. Mouse embryo fibroblasts (Specialty Media, Phillipsburg, NJ; cat. no. PMEF-HL).
2. D3 ES cells *(7,8)*.
3. ES cells genetically deleted for the fibronectin and α5-integrin genes were generated as described by Robinson *(7,8)*.
4. ES cells lacking β3-integrin were generated in-house from heterozygous ES cells according to the method of Mortensen et al. *(9)*.
5. 1X phosphate-buffered saline (PBS) (Gibco, Carlsbad, CA; cat. no. 18912-014).
6. Fetal calf serum (FCS) (Hyclone Inc., Logan, UT; cat. no. SH30070.03E).
7. High-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco, cat. no. 41966).
8. DMEM HEPES medium (Gibco, cat. no. 42430).
9. Iscove's modified Dulbecco's medium (Gibco, cat. no. 21980).
10. Glutamine (Gibco, cat. no. 25030-024).
11. Nonessential amino acids (NEAA) (Gibco, cat. no. 11140-035).
12. 100 m*M* minimum essential medium sodium pyruvate (Gibco, cat. no. 11360-039).
13. Penicillin-streptomycin (Gibco, cat. no. 15070-063).
14. Trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco, cat. no. 25300-054).
15. Corning polystyrene 100-mm tissue culture dishes (Corning, Acton, MA).
16. Bacterial-grade Petri dishes (3.5 mm).
17. 0.2-μm syringe filters.
18. Vascular endothelial growth factor (VEGF) (Peprotech, Rocky Hill, NJ; cat. no. 450-32).
19. Methylcellulose (base) (Stem Cell Technologies, Vancouver, Canada; cat. no. 03120).
20. Mouse interleukin (IL)-6 (Peprotech, cat. no. 450-32).
21. Monothioglycerol (MTG) (Sigma, St. Louis, MO; cat. no. M6145) (*see* **Note 1**).
22. LIF (ESGRO 1000 U/mL) (Gibco, cat. no. 13275).
23. G418 (Gibco, cat. no. 10131-019).
24. Mouse erythropoietin (Sigma, cat. no. E9530).
25. Insulin (Sigma, cat. no. I0516).
26. Fibroblast growth factor (FGF)-2 (Peprotech, cat. no. 100-18).

2.1.1. Media

1. Medium for mouse embryonic fibroblast (MEF) cells: MEFs are maintained in 1X DMEM HEPES medium supplemented with 2 m*M* glutamine, 10% v/v FCS, 1 m*M* sodium pyruvate, and 0.1 m*M* NEAA. To a 500-mL bottle of DMEM, add 50 mL FCS, 10 mL sodium pyruvate, 5 mL glutamine, and 1 mL NEAA.

2. Medium for ES cells: D3 ES cells are maintained in supplemented 1X high-glucose DMEM HEPES supplemented with 2 m*M* glutamine, 0.1 m*M* NEAA, 100 µ*M* MTG, 100 U/mL penicillin-10 µg/mL streptomycin, 10 ng/mL LIF, and 15% v/v FCS. To a 500-mL bottle of DMEM, add 150 mL FCS, 10 mL sodium pyruvate, 5 mL glutamine, 0.1 mL NEAA, 5 mL penicillin-streptomycin, 6.3 µL MTG, and 45 µL LIF.
3. Medium for homozygous null ES cells from heterozygous ES cells: G418 dissolved in 0.1 *M* HEPES, at pH 7.2 and sterile filtered; stock concentration 50 mg/mL. For a 5 mg/mL solution of G418 in ES medium (*see* **step 2**), add 2 mL stock G418 to 20 mL complete ES media.
4. For mitomycin C/MEF medium, add 2 mg mitomycin C (sterile filtered) to 200 mL MEF medium. Store at 4°C and use this medium within 4 wk.

2.1.2. General Comments and Equipment

Tissue culture procedures must be performed using sterile techniques and without antibiotics if possible. All solutions should be warmed to 37°C. It is important to avoid contamination, so dedicated culture and incubation facilities are desirable. Facilities require a 37°C water bath for warming media, a humidified incubator at 37°C and 5% CO_2, an inverted microscope, a class II microbiological safety cabinet, a refrigerator and freezer facilities, and a bench centrifuge for 15- and 50-mL tubes.

2.2. Whole-Mount Immunofluorescence Staining of Vascular Networks in EBs

1. Dimethyl sulfoxide.
2. Methanol.
3. CD31 rat antimouse platelet-EC adhesion molecule (PECAM), MEC 13.3 (Pharmingen, San Diego, CA; cat. no. 553372).
4. Secondary goat antirat fluorescein isothiocyanate (Biosource, Camarillo, CA; cat. no. ARI4408).
5. Bovine serum albumin (BSA) (Sigma, cat. no. A9576).
6. PBS (Gibco, cat. no. 18912-014).
7. 50% MeOH/PBS.
8. 2% w/v BSA/0.1% v/v Tween-20 in PBS.
9. Antifade mounting reagent (Bio-Rad, Hercules, CA; cat. no. 170-3140).
10. MRC Bio-Rad 1024 confocal microscope (Bio-Rad).

3. Methods

*3.1. Tissue Culture (***see** ***Note 2)***

It is important not to keep ES cells in culture for extended periods or to allow ES cells to become overconfluent. These practices encourage abnormal karyotypes and promote premature or inconsistent differentiation. Generally, ES cells that have spent fewer than 10 d in tissue culture prior to differentiation produce the best results. Undifferentiated ES cells have a large nucleus with very little cytoplasm. It is difficult to identify individual cells in the colony as there are nondistinct cytoplasmic membranes between the cells. Colonies are amorphous and of different size. When differentiation occurs, cytoplasmic membranes between the cells become apparent. The colonies spread and flatten.

*3.1.1. MEF Feeder Layers (*see ***Note 3****)*

MEFs should be mitotically inactivated so that they do not overgrow the ES cell cultures. This may have already been done by irradiation (60–100 cGy) or treatment with

mitomycin C (10 µg/mL for 3 h). Mitomycin C treatment is easier if an irradiator is not readily available. Use the treated MEFs within 10 d (*see* **Note 4**).

1. For MEFs that have not been mitotically inactivated, remove the medium from the cells in the flask.
2. Replace the medium with mitomycin C in MEF medium (*see* **Subheading 2.1.1.**) to cover the cells in a layer a few millimeters deep.
3. Incubate at 37°C for 2–3 h.
4. Aspirate the mitomycin C/MEF medium.
5. Wash three times with PBS.
6. Cells can be passaged in the usual manner and seeded as required.

3.1.2. Generation of Homozygous ES Cells From Heterozygous ES Cells

This method is useful for the generation of a homozygous null clone from a heterozygous clone and relies on the ability of high concentrations of G418 to cause mutation of an additional targeted allele. It has been adapted from the work of Mortensen et al. ***(9)***.

1. Plate heterozygous ES cells on feeder fibroblasts in the usual manner.
2. Split ES cells in a 1:6 ratio and plate in duplicate in new dishes on feeder cells in medium containing 0–5 mg/mL G418.
3. Change the medium on the ES cells on d 1, 2, 3, 4, 6, and 7 and 2 h prior to picking clones with media containing the ascribed concentration of G418.
4. Pick visible ES clones on the 2- and 3-mg/mL plates after 7 d and replate in selective medium (*see* **Note 5**).
5. Freeze clones; take with some of the clone for DNA analysis to check for mutation of the other specific allele.

3.1.3. Differentiation of ES Cells Into EB: Promotion of Endothelial Cell Development

To avoid artifacts caused by differentiation on plastic dishes, ES cells are differentiated in a three-dimensional organ culture-type system as described by Vittet et al. ***(10)***. This is a methylcellulose-based system supplemented with endothelial cell differentiation factors. Cells are seeded at very low densities in methylcellulose in 3.5-mm diameter bacterial-grade dishes, and these differentiate over the course of 28 d into EBs containing an inner vascular network ***(10,11)***. Low-passage ES cells work best. Cells should be undifferentiated (not spread, but exhibiting round, "plump" ES cell morphology). Viability should be 90% or better. It is important to remove as many feeders as possible from the cells to be seeded using a differential plating technique. If too many cells are seeded, then they will become crowded, contact other EBs, and become prematurely attached to the base of the culture dish. It is also important to maintain the dishes in a local humidified environment.

EBs can be viewed within the methylcellulose under phase contrast microscopy. They undergo several stages of differentiation, as shown in **Fig. 1**. The first sign of an EB is a dense mass of cells surrounded by a cellular envelope. Other cell types form at the edge of the EB, and a primitive form of hemoglobin may form (**Fig. 1**).

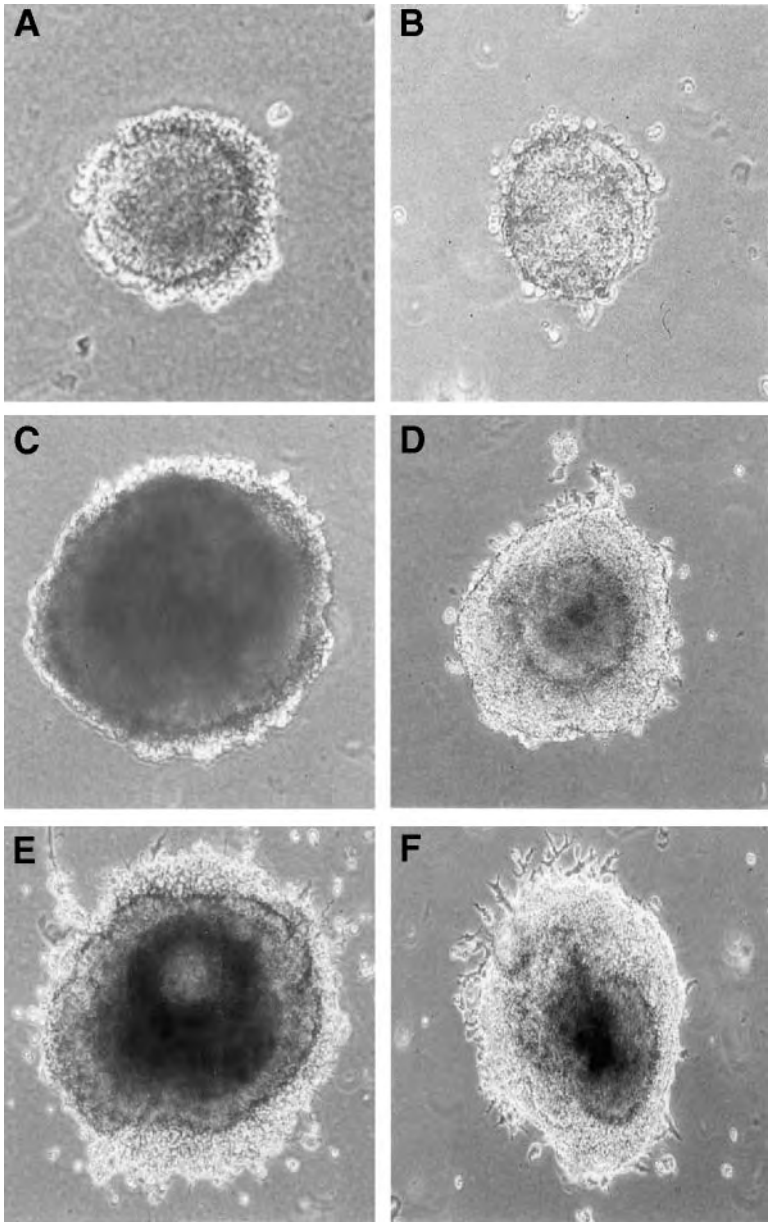

Fig. 1. Embryoid bodies (EB) viewed under phase contrast microscopy at various times after the onset of differentiation. (**A**) Wild-type (control) 5-d EB; (**B**) fibronectin (FN) null 8-d EB; (**C**) wild-type (control) 8-d EB; (**D**) FN null 8-d EB; (**E**) wild-type (control) 11-d EB; (**F**) FN null 11-d EB. Magnification ×400 before reproduction. Observe the lack of integrity of the FN null EBs at d 8 and 11; this is characteristic of FN null EB development.

3.1.3.1. Preparation of Methylcellulose

Methylcellulose is a glutinous preparation that requires dilution before use for differentiation.

1. Thaw the methylcellulose at 37°C.
2. Aliquot the required amount (final concentration 1% w/v) into a separate tube using a 10-mL syringe attached to an 18-gage blunt-end needle.
3. Dilute the methylcellulose with Iscove's medium containing the required factors (*see* **Note 6**).

3.1.3.2. Seeding ES Cells Into Methylcellulose

Always use cells that are of low passage number and that have been kept in an undifferentiated state (preferably not more than 10 d in culture). Before the ES cells are seeded into methylcellulose, the feeder cells need to be removed via a differential plating technique. Basically, this involves plating a mixed population of feeders and ES cells onto gelatin-coated tissue culture-grade dishes for 30 min only. This allows the feeder cells to attach, leaving the ES cells in the supernatant or just loosely attached. ES cells need to be pipeted off to be used in the differentiation procedure.

1. Gelatin coat two 10-cm tissue culture dishes.
2. Rinse one dish of ES cells well with prewarmed PBS.
3. Add 2 mL trypsin-EDTA for 5 min at 37°C.
4. Stop the trypsin with 2 mL media containing serum or 100 µL neat serum.
5. Disperse all the cells using a sterile transfer pipet.
6. Add culture medium without LIF to 10 mL medium, centrifuge at room temperature at 500*g* for 2 min.
7. Remove the supernatant and resuspend cells in 2 mL medium without LIF and plate on a 10-cm dish.
8. Incubate for 30 min at 37°C, during which time the fibroblasts will attach.
9. Remove the supernatant and wash the cell layer gently with PBS. The majority of these cells are ES cells only. Any fibroblasts still remaining will be unable to proliferate.
10. Seed 2×10^3 cells into 1×3.5-mm diameter dish containing methylcellulose and endothelial growth factors at the following concentrations:

FCS	15% w/v
FGF-2	100 ng/mL
Erythropoeitin	2 U/mL
Insulin	10 µg/mL
VEGF	50 ng/mL
MTG (fresh)	450 µ*M*
IL-6	10 ng/mL

11. Place the dishes (with lids) inside a humidified chamber made from a plastic lunchbox containing an open Petri dish containing sterile water. Replenish the sterile water every 3 d.

3.1.3.3. Feeding EBs

EBs should be fed every 5 d while in culture.

1. Prepare 0.5% w/v methylcellulose with the required added growth factors.
2. Add carefully dropwise using a syringe attached to a blunt-end 18-gage needle onto the surface of the methylcellulose already in the dish.

3.1.2.4. Removal of EBs From Methylcellulose

When the EBs are large enough (approx 11 d in culture), they will form almost-round entities within the methylcellulose and will be visible with the naked eye or under the inverted phase contrast microscope (**Fig. 1**). In most cases, if suspended in the methylcellulose and treated gently, then they will be robust enough to be washed out of the gel and manipulated using plastic transfer pipets.

1. Carefully add warm PBS to the Petri dish with a transfer pipet.
2. Flush the methylcellulose and PBS into a 50-mL polystyrene conical tube; mix gently.
3. Add additional warm PBS (as much as 20 mL may be required) and gently manipulate the mixture until the EBs are free. They will sink to the bottom of the tube if left for 5–10 min.
4. Alternatively, EBs can be centrifuged out of the methylcellulose at 300*g* for 10 min. The EBs form a loose pellet.
5. According to the age of the EBs, disrupt them with either trypsin-EDTA (<8-d-old EBs) or collagenase (<8-d-old EBs).
   a. For EBs less than 8 d old, add trypsin for 2–3 min at room temperature. Disrupt the EBs by passing through a 20-gage needle on a 5-mL syringe to obtain a single-cell suspension. Neutralize the trypsin with media containing FCS.
   b. For EBs older than 8 d, add collagenase for 1 h at 37°C. Disrupt EBs as above. Neutralize the trypsin with media containing FCS.

3.2. Analysis of Differentiation of Vascular Networks by Confocal Microscopy

Analysis of differentiation of vascular networks is an important technique that allows visualization of the entire vascular network using optical confocal imaging and reconstruction. EBs are permeabilized, fixed, and labeled before visualization using a water immersion lens on a confocal microscope. A motorized stage capable of taking 0.5-µm or smaller z-section images allows reconstruction of multiple stacked images and displaying either as a two-dimensional stack or three-dimensional analglyph. Most confocal microscopes have a z-section facility and will allow reconstruction of stacked images to create montages or movies.

3.2.1. Whole-Mount Staining Procedure

1. Harvest EBs gently from the MCM (*see* **Subheading 3.1.2.4.**).
2. Place in a 1-mL Eppendorf tube with dimethyl sulfoxide:methanol at a 1:4 volume ratio overnight at 4°C to permeabilize and fix. The tube should be put on a tube rotator or roller if possible.
3. Wash the EBs at least four times in 50% MeOH/PBS. To wash the EBs, remove from the rotator and allow to settle (approx 10 min), aspirate the solution from the tube, and add fresh wash medium.

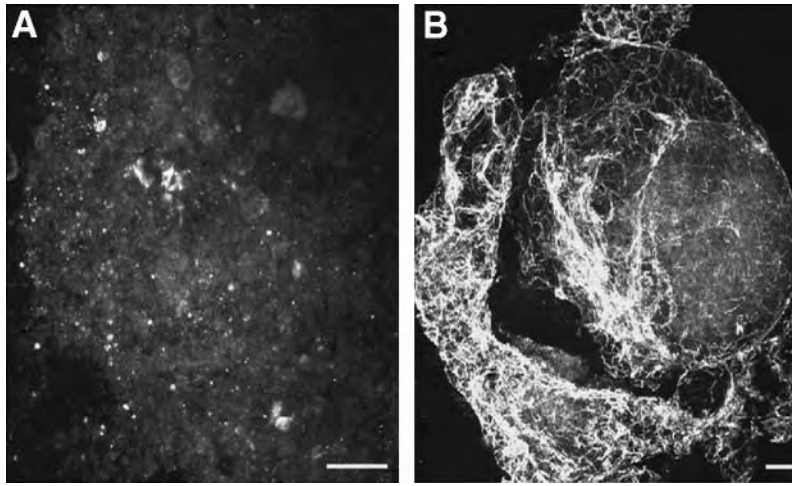

Fig. 2. Two-dimensional representation of a series of 40 confocal images (stacked) from (**A**) an embryoid bodies (EB) at 11 d without endothelial growth-promoting factors and (**B**) an EB at 11 d with endothelial growth-promoting factors. Scale bar denotes 50 µm.

4. Preincubate in blocking solution of 2% w/v BSA/0.1% v/v Tween-20 in PBS for 30 min at room temperature while continually rotating.
5. Incubate with rat anti-mouse PECAM 1:100 in blocking solution for between 6 and 12 h at room temperature. Keep the samples rotating slowly.
6. Perform four 15-min washes with 2% w/v BSA/0.1% v/v Tween/PBS.
7. Incubate with a fluorescein isothiocyanate-conjugated secondary antibody 1:200 in blocking solution for 2 h at room temperature. Keep the samples rotating slowly.
8. Wash the EBs at least three times in 2% w/v BSA/0.1% v/v Tween/PBS.
9. Wash once in PBS.
10. Mount in antifade mounting medium either gelvatol or commercial antifade mountant on a chamberslide with a well so the EBs are not squashed.

3.2.2. Confocal Microscopy of EBs

Although EBs can be embedded in gelatin and frozen sections prepared and stained, this is difficult and only gives a "snapshot" of the vascular network at any one time. With the advent of nondestructive confocal imaging techniques, the network within the EB can be seen using optical sectioning and can be reconstructed by computer modeling. This can be done from a series of stacked image slices and is likely to be possible from within the confocal imaging software itself. It is important to make very thin slices (<0.5 µm) through the EBs so pixels, and therefore data, are not lost.

Figure 2 shows the vascular network in EBs cultured with and without growth factors. In **Fig. 3**, a small area of co-localized PECAM and α-smooth muscle cell actin staining of EC in an α5-null EB can be clearly seen in a single optical slice. In this montage, few ECs can be seen (bright area); this is characteristic of α5-null EBs. The other stained cells in the rest of the EBs are stained only for α-smooth muscle actin. Slices

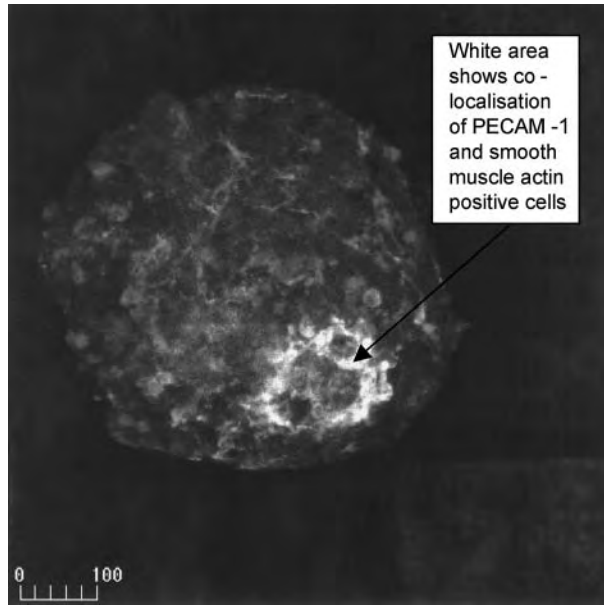

Fig. 3. A 1-µm thick optical slice of an α5-integrin null embryoid bodies viewed using a confocal microscope using dual-color labeling (α-smooth muscle cell actin and platelet-endothelial cells adhesion molecule-1-positive cells co-localize in the bright circular area). Scale bar denotes 100 µm

can be stacked and presented as one two-dimensional image (as in **Fig. 2**) or viewed as individual slices (**Fig. 3**).

4. Notes

1. It is important that the diluted MTG is freshly made.
2. Adopt standard ES protocols when freezing and thawing cells. In addition, generally freeze cells slowly and thaw cells rapidly. For long-term storage, keep ES cells at –135°C or under liquid nitrogen.
3. It can be easier, but more expensive, to purchase quality-controlled mitotically inactivated MEFs for feeder layers rather than to prepare them in-house. MEFs have a relatively short life-span, so it is also advisable to have a stock of frozen cells ready to use when needed. MEFs (neomycin resistant and mitomycin C treated) can be purchased from www.specialtymedia.com.
4. Try to keep the MEFs actively dividing prior to mitotic activation, ideally by passaging the day before treatment and use. Once MEFs are mitotically inactivated by whatever means, they can be used as feeders for up to 10 d (about passage 4). After this point, their ability to support the undifferentiated growth of ES cells wanes as they begin to senesce.
5. In the early phases, ES cell death is visible only on the 4- and 5-mg/mL G418-treated plates. Usually, by d 7 no ES cells are visible on the high G418 containing-medium plates.
6. Take care not to introduce bubbles into the methylcellulose as this will affect differentiation and viewing of the EBs using microscopy. If you do introduce bubbles, let them settle out for about 20 min before adding any cells.

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23

TGF-β Signaling in Embryonic Stem Cell-Derived Endothelial Cells

Tetsuro Watabe, Jun K. Yamashita, Koichi Mishima, and Kohei Miyazono

Summary

The roles of the transforming growth factor (TGF)-β superfamily in vasculogenesis have been implicated by the findings that mutations in genes encoding for various TGF-β superfamily signaling components exhibit defects in vascular tissues in humans and mice. Embryonic stem cell (ESC)-derived vascular progenitor cells have been shown to differentiate into both endothelial and mural cells. We showed that members of the TGF-β superfamily play important roles during differentiation of vascular progenitor cells derived from mouse ESC. TGF-β inhibited proliferation and sheet formation of ESC-derived endothelial cells. Interestingly, SB-431542, a synthetic molecule that inhibits the kinases of receptors for TGF-β and activin, facilitated proliferation and sheet formation of ESC-derived endothelial cells. We also found that stimulation of ESC-derived endothelial cells with TGF-β resulted in phosphorylation of both Smad2 and Smad1/5; BMP induced phosphorylation of Smad1/5. In this chapter, we present how to study the cellular and biochemical effects of TGF-β signals on endothelial cells derived from mouse ESCs.

Key Words: Activin; BMP; endothelial development; Flk-1; PECAM-1; phosphorylation; SB-431542; Smad; α-smooth muscle actin; vascular progenitor cells; Western blot.

1. Introduction

1.1. Differentiation of Endothelial Cells in Embryos and In Vitro Differentiation System

Embryonic vessels are thought to be formed by endothelial cells that arise from fetal liver kinase (Flk)-1 (vascular endothelial growth factor [VEGF] receptor 2)-expressing (Flk-1+) mesoderm cells, surrounded by mural cells (pericytes and vascular smooth muscle cells) derived from mesoderm, neural crest, or epicardial cells. These embryonic processes, referred to as vasculogenesis, contrast with angiogenesis, the term applied to the postnatal neovascularization that results from the proliferation and remodeling of differentiated endothelial cells from preexisting vessels

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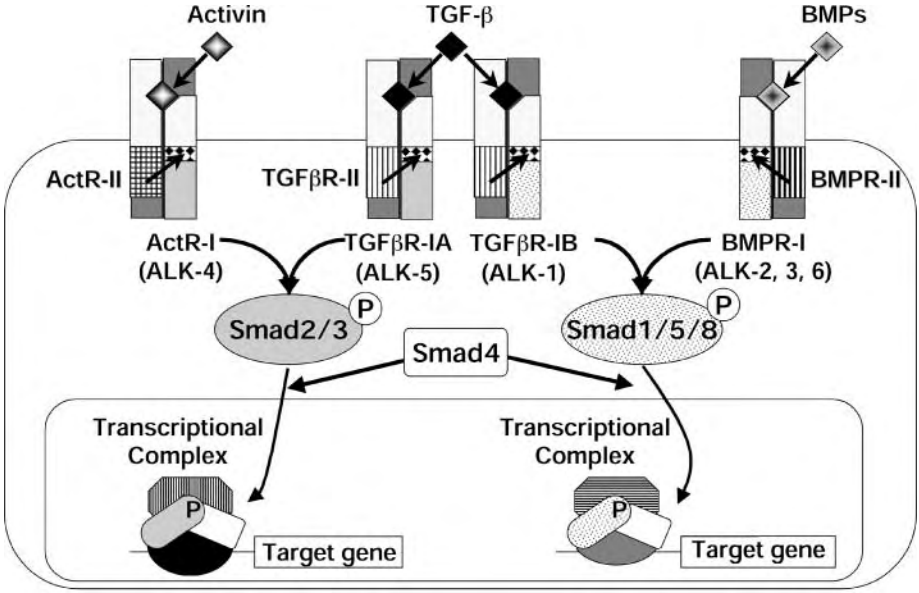

Fig. 1. Signal transducing pathways mediated by transforming growth factor (TGF)-β superfamily members. ActR-I, activin type I receptor; ActR-II, activin type II receptor; TβR-I, TGF-β type I receptor; TβR-II, TGF-β type II receptor; BMPR-I, BMP type I receptor; BMPR-II, BMP type II receptor; P, phosphorylated.

(1). Embryonic stem cell (ESC)-derived vascular progenitor cells have been shown to differentiate into both mural and endothelial cells *(2–4)*. Interaction between endothelial and mural cells plays important roles in development of vascular tissues and maintenance of their homeostasis in both embryonic and adult tissues *(5)*. Transforming growth factor (TGF)-β superfamily members have been implicated as cytokines that serve such interaction *(6)*.

1.2. Signal Transduction by TGF-β Superfamily Members in Endothelial Cells

Cytokines of the TGF-β superfamily are dimeric proteins with conserved structures and have a broad array of biological activities *(7)*. The TGF-β superfamily includes nearly 30 proteins in mammals, such as TGF-βs, activins, Nodal, and bone morphogenetic proteins (BMPs). TGF-βs are synthesized as latent high molecular weight complexes from producer cells and are activated by various mechanisms, including effects of plasmin and thrombospondin. Active TGF-β then binds to heteromeric complexes of type II and type I serine/threonine kinase receptors (**Fig. 1**). On ligand binding, the constitutively active type II receptor kinase phosphorylates the type I receptor, which in turn activates the downstream signal transduction cascades, including Smad pathways.

Although all members of the TGF-β superfamily utilize essentially similar signaling pathways, the signals converge into two types of intracellular Smad signals. Activins

and TGF-βs bind to type I receptors known as activin receptorlike kinase (ALK) 4 and 5, respectively. The activated type I receptors phosphorylate receptor-regulated Smad proteins (R-Smads). Smad2 and 3 transduce signals for TGF-βs and activins; Smad1, 5, and 8 are specific for signaling of BMPs, which bind to ALK-2, -3, and -6 *(8)*. As an exception, ALK-1, preferentially expressed in endothelial cells, binds TGF-β and activates Smad1/5 pathways *(9)*.

The roles of the TGF-β superfamily in vasculogenesis have been suggested by the findings that knockout mice deficient in various TGF-β superfamily signaling components (e.g., ALK-1, endoglin, Smad5) exhibit defects in vascular tissues *(10)*. Moreover, heterozygous mutations in human genes coding for endoglin, an accessory protein for the TGF-β receptor complex, or ALK-1 cause a human hereditary vascular syndrome, hereditary hemorrhagic telangiectasia *(11)*. However, the lack of in vitro systems consisting of both endothelial and mural cell populations has hampered dissection of the roles of TGF-β superfamily signaling in vascular development.

1.3. Roles of TGF-β Superfamily Signals in ESC-Derived Endothelial Cells

To elucidate the roles of TGF-β superfamily signaling in vascular differentiation from their progenitors, we used in vitro differentiation systems derived from mouse ESCs *(12)*. According to a previous report *(2)*, undifferentiated mouse ESCs were cultured to induce Flk-1+ cells, which were purified by cell sorting using anti-Flk-1 antibody. After an additional 3 d of culture of Flk-1+ cells in the presence of VEGF, we were able to obtain platelet-endothelial cell adhesion molecule (PECAM)-1-positive sheets of endothelial cells. The remaining cells surrounding the sheets were positive for a mural cell marker, α-smooth muscle actin (SMA) (**Fig. 2A**). We next examined the effects of the TGF-β superfamily proteins on the in vitro vascular differentiation of ESC-derived Flk-1+ cells. Although BMP did not exhibit significant effects (**Fig. 2B**), TGF-β led to the decrease in PECAM-1+ sheets of endothelial cells (**Fig. 2C**). SB-431542, a synthetic molecule that inhibits the kinases of receptors for TGF-β and activin *(13,14)*, facilitated proliferation and sheet formation of ESC-derived endothelial cells (**Fig. 2D**). These results suggest that endogenous TGF-β/activin signals play important roles in regulating vascular growth and permeability.

1.4. Activation of R-Smads by TGF-β Superfamily Signals in ESC-Derived Vascular Cells

We also studied the biochemical effects of endogenous and exogenous TGF-β superfamily members on phosphorylation of R-Smads using a phospho-Smad1/5 antibody and a phospho-Smad2 antibody. Stimulation of ESC-derived vascular cells with TGF-β for 1 h resulted in phosphorylation of both Smad2 and Smad1/5 (**Fig. 3**, **lane 3**), as previously reported in other types of primary endothelial cells *(15)*. BMP7 led to phosphorylation of only Smad1/5 (**Fig. 3**, **lane 5**). To evaluate whether TGF-β-induced Smad1/5 phosphorylation is specific to endothelial cells, we analyzed phosphorylation of Smads by TGF-β in ESC-derived mural cells. Although TGF-β induced Smad2 phosphorylation in mural cells (**Fig. 3**, **lane 8**), it failed to phosphorylate Smad1/5. These results suggest that TGF-β induced phosphorylation of both Smad2 and Smad1/5 only in ESC-derived endothelial cells.

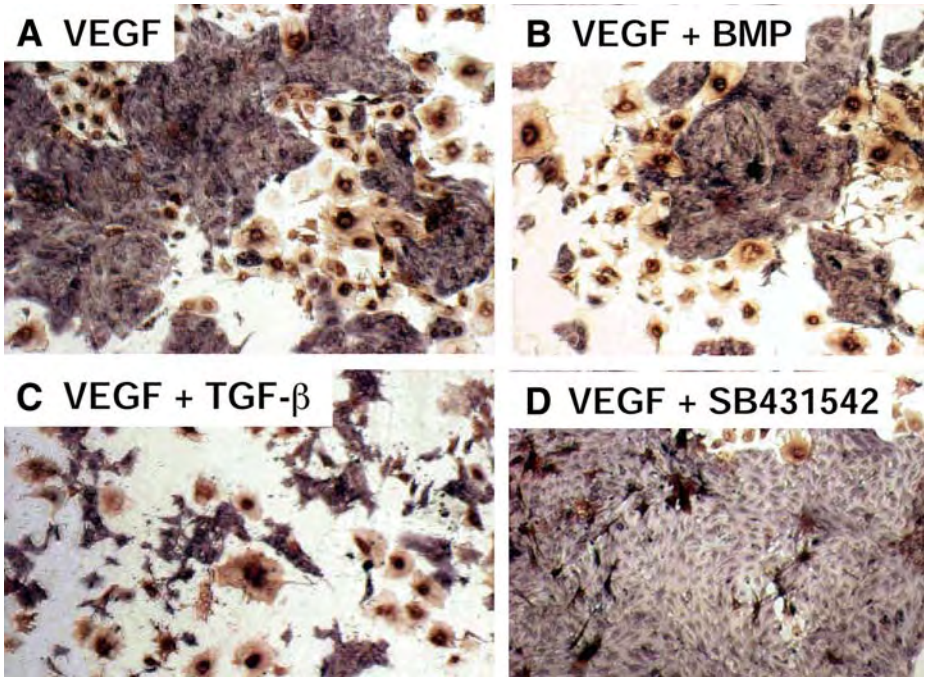

Fig. 2. Effects of transforming growth factor (TGF)-β superfamily members on differentiation of embryonic stem cells (ESC)-derived Flk-1+ cells into endothelial and mural cells. (**A**) Platelet-endothelial cell adhesion molecule-1 (purple) and α-smooth muscle actin (1A4; brown) immunostaining of CCE cell-derived vascular cells. ESC-derived Flk-1+ cells were treated with vascular endothelial growth factor in the presence of 10% fetal calf serum. The cells were also treated with (**B**) BMP7, (**C**) TGF-β, or (**D**) SB431542. (Please *see* the companion CD for the color versions of this figure.

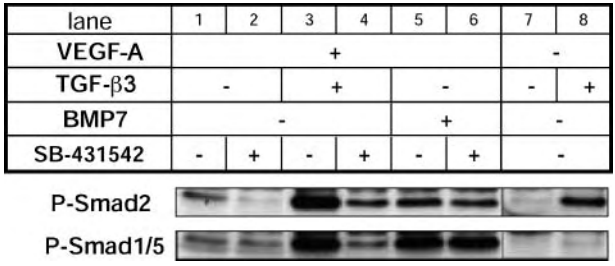

Fig. 3. Transforming growth factor (TGF)-β-induced phosphorylation of Smad2 and Smad1/5 in embryonic stem cell (ESC)-derived endothelial cells. Phosphorylation of R-Smads by TGF-β superfamily members in ESC-derived endothelial and mural cells. Flk-1+ cells were cultured with 10% FCS and vascular endothelial growth factor for 3 d to induce differentiation into endothelial and mural cells and treated with TGF-β or BMP7 in the absence or presence of SB-431542 for 1 h. Mural cells differentiated from Flk-1+ cells with 10% FCS were treated with TGF-β for 1 h. Cell lysates were directly subjected to Western blot analysis using phospho-Smad2 (**top panel**) and phospho-Smad1/5 (**bottom panel**) antibodies.

When SB-431542 was added in the presence of TGF-β, Smad2 phosphorylation was decreased because of its inhibitory effect on ALK-5 (**Fig. 3**, **lane 4**). We found that Smad1/5 phosphorylation induced by TGF-β was also decreased by SB-431542 (**Fig. 3**, **lane 4**). Because SB-431542 is not capable of inhibiting Smad phosphorylation by ALK-1 ***(13,14)***, these results suggest that ALK-5 kinase activity is required for ALK-1-mediated phosphorylation of Smad1/5 in ESC-derived endothelial cells. Inhibition of Smad1/5 phosphorylation by SB-431542 is specific for ALK-1 because Smad1/5 phosphorylation by BMP was not decreased by SB-431542 (**Fig. 3**, **lane 6**).

Importantly, phosphorylation of Smad2 was weakly detected in the absence of exogenous ligands (**Fig. 3**, **lane 1**), suggesting that endogenous TGF-β or activin acts on these cells. TGF-βs are released from cells in latent forms consisting of the mature growth factors associated with amino-terminal propeptides and latent TGF-β-binding proteins ***(16)***. Endogenous activation of latent TGF-β has been shown to occur efficiently in co-cultures of endothelial and smooth muscle cells through activation of plasmin on the cell surface, suggesting that endogenous TGF-β may regulate the functions of ESC-derived vascular cells. Moreover, addition of SB-431542 resulted in the decrease of phospho-Smad2 in these cells (**Fig. 3**, **lane 2**). These results strongly implicate the causal link between the states of Smad2 phosphorylation induced by TGF-β signals and endothelial sheet formation.

2. Materials

2.1. ESC Lines

Although numerous ESC lines are currently available, each line should be examined for its ability to generate Flk-1+ cells. The ESC line most commonly used in our laboratories is CCE ***(17)***. The methods presented here are optimized for CCE cells (*see* **Note 1**).

2.2. Reagents

2.2.1. ESC Differentiation Medium

1. Minimum essential medium α medium (Gibco BRL, Grand Island, NY; cat. no. 12000-022).
2. Penicillin-streptomycin (Gibco BRL, cat. no. 15070-063).
3. Fetal calf serum (FCS) (Equitech-Bio Inc., Kerrville, TX; lot. no. SFB30-1201) (*see* **Note 2**).
4. 2-Mercaptoethanol (Gibco BRL; cat. no. 21985-023).
5. ESC differentiation medium: for 1 L, add 10.1 g minimum essential medium α medium powder and 2.2 g $NaHCO_3$ to 800 mL H_2O and stir using a stirrer and stir bar until the solution is clear. Add 100 mL FCS, 1 mL 2-mercaptoethanol, and 20 mL penicillin-streptomycin and bring final volume to 1 L with water. Mix well and sterilize using a 0.22-μm filter unit (Millipore Corp., Bedford, MA; cat. no. SLGP033RB).

2.2.2. Differentiation and Purification of Flk-1+ Cells

1. Collagen type IV-coated 10-cm plates, Biocoat (Beckton Dickinson, Franklin Lakes, NJ; cat. no. 354453).
2. Phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} (Gibco BRL, cat. no. 14190-144).
3. Trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco BRL, cat. no. 25200-056).

4. MACS solution: for 50 mL, add 0.2 mL 0.5 *M* EDTA (pH 7.4) and 25 mg bovine serum albumin (Sigma Aldrich, St. Louis, MO; cat. no. A4503) to 50 mL PBS in a 50-mL Falcon tube. Mix well and sterilize using a 0.22-µm filter unit (*see* **Subheading 2.2.2., item 4**).
5. Phycoerythrin (PE)-conjugated anti-mouse Flk-1 antibody (AVAS12) (eBioscience, San Diego, CA; cat. no. 12-5821-83).
6. Anti-PE microbeads (Miltenyl Biotec, Bergisch Gladbach, Germany; cat. no. 130-048-801).
7. MACS separation column (Miltenyl Biotec, cat. no. 130-042-201).

2.2.3. Differentiation of Endothelial Cells From ESC-Derived Flk-1+ Cells

1. Collagen type IV-coated 24-well plates, Biocoat (Beckton Dickinson; cat. no. 354430).
2. Recombinant human $VEGF_{165}$ (R&D Systems, Minneapolis, MN; cat. no. 293-VE-010).

2.2.4. Treatment of ESC-Derived Vascular Cells With TGF-β Superfamily Ligands and Chemicals

1. TGF-β3 (R&D Systems, cat. no. 243-B3-002) (*see* **Note 3**).
2. Recombinant human BMP7 (R&D Systems, cat. no. 314-BP-010) (*see* **Note 3**).
3. SB431542 (inhibitor of ALK-4/-5/-7 receptors) (Sigma Aldrich, cat. no. S4317): prepare stock solution (10 m*M*) in dimethyl sulfoxide (Sigma Aldrich, cat. no. D2650).

2.2.5. Staining of ESC-Derived Vascular Cells for Vascular Markers

1. Mouse anti-PECAM-1 antibody (Mec13.3) (BD Pharmingen, San Jose, CA; cat. no. 553370).
2. Rat anti-SMA antibody (1A4) (Sigma Aldrich, cat. no. A2547).
3. Horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin (Ig)G (Zymed, San Francisco, CA; cat. no. 62-6520).
4. Alkaline phosphatase (AP)-conjugated goat anti-rat IgG (Zymed, cat. no. 62-9522).
5. 4% paraformaldehyde (PFA): for 100 mL, using a stirrer/hot plate and stir bar, heat 60 mL PBS to 65°C. Add 4 g PFA and stir. Add 60 µL 0.1 *N* NaOH and stir until the solution is clear; remove from heat. Bring final volume to 100 mL with PBS. Mix well and pour into two 50-mL Falcon tubes; cool on ice for at least 20 min.
6. 0.3% H_2O_2/methanol: for 100 mL, add 1 mL 30% H_2O_2 (Sigma Aldrich, cat. no. H1009) to 99 mL methanol and mix well.
7. 2% skim milk/PBS.
8. PBS containing 0.05% Tween-20 (PBS-T) (Wako Chemicals, Osaka, Japan; cat. no. 163-11512).
9. Histofine simple stain diaminobenzidine (DAB) solution (Nichirei, Tokyo, Japan; cat. no. 415171).
10. AP solution: for 100 mL, add 10 mL 1 *M* Tris-Cl (pH 9.5), 0.6 g NaCl, 1 g $MgCl_2$ to 80 mL H_2O and stir until the solution becomes clear. Bring final volume to 100 mL with water and mix well. Add 0.5 mL 400 m*M* Levamisole stock (Sigma Aldrich, cat. no. L9756) before use.
11. 4-Nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) (Roche, Indianapolis, IN; cat. no. 1-681-451).

2.2.6. Western Blot Analysis of ESC-Derived Vascular Cells

1. Lysis buffer: 25 m*M* Tris-HCl at pH 7.5, 137 m*M* NaCl, 0.5% Triton X-100, 1% aprotinin, and 1 m*M* phenylmethylsulfonyl fluoride. For 1 L, dissolve 3 g Tris-base and 8 g NaCl in 600 mL H_2O. Adjust to pH 7.5 with 1 *N* HCl and add 5 mL Triton X-100. Bring final volume to 1 L with water and mix well. Add aprotinin and phenylmethylsulfonyl fluoride before use.
2. PBS (*see* **Subheading 2.2.2., item 2**).

3. 4X Tris-HCl/sodium dodecyl sulfate (SDS): for 100 mL, dissolve 6.05 g Tris-base in 40 mL H_2O. Adjust to pH 6.8 with 1 *N* HCl. Bring final volume to 100 mL with water. Add 0.4 g SDS and mix well.
4. SDS sample buffer: for 10 mL, mix 7 mL 4X Tris-HCl/SDS (*see* **Subheading 2.2.6., item 3**), 3 mL glycerol, 1 g SDS, 0.93 g dithiothreitol, and 1.2 mg bromophenol blue. Bring the final volume to 10 mL with water if needed. Store in 0.5-mL aliquots at –70°C.
5. Transfer membrane (Pall Corp., Easr Hills, NY; cat. no. EH-2222).
6. Electrode paper (Amersham Biosciences, Uppsala, Sweden; cat. no. 80-1106-19).
7. Transfer buffer, anode 1:300 m*M* Tris-base, 20% methanol. For 1 L, dissolve 36 g Tris-base in 600 mL H_2O and bring the final volume to 800 mL with water. Add 200 mL methanol and mix well.
8. Transfer buffer, anode 2 (25 m*M* Tris-base, 20% methanol): for 1 L, dissolve 3 g Tris-base in 600 mL H_2O and bring the final volume to 800 mL with water. Add 200 mL methanol and mix well.
9. Transfer buffer, cathode: 40 m*M* ε-amino-caproic acid, 20% methanol. For 1 L, dissolve 5.2 g ε-amino-caproic acid in 600 mL H_2O and bring the final volume to 800 mL with water. Add 200 mL methanol and mix well.
10. Tris-buffer saline: 25 m*M* Tris-HCl, 137 m*M* NaCl, 2.68 m*M*, pH 7.4 (TBS). For 1 L, dissolve 3 g Tris-base, 8 g NaCl, and 0.2 g KCl in 600 mL H_2O. Adjust to pH 7.4 with 1 *N* HCl. Bring final volume to 1 L with water and mix well.
11. TBS containing 0.05% Tween-20 (TBS-T).
12. 5% skim milk/TBS-T.
13. Anti-phospho-Smad2 antibody (Cell Signaling Technology, Beverly, MA; cat. no. 3101).
14. Anti-phospho-Smad1/5 antibody (Cell Signaling Technology, cat. no. 9511).
15. HRP-conjugated anti-rabbit IgG (Amersham Biosciences, cat. no. NA934).

3. Methods

3.1. Differentiation of Endothelial Cells From ESCs

This method is a modified version of previously described protocol *(18)*.

1. Plate 1×10^5 undifferentiated ESCs into each 10-cm collagen type IV-coated plate containing 15 mL differentiation medium.
2. Leave undisturbed for 4–5 d in a 37°C incubator with 5% CO_2 environment (*see* **Note 4**).
3. After 4–5 d, remove the medium, wash with PBS twice, and incubate at 37°C with 0.5 mL trypsin/EDTA for 5 min. Harvest cells by pipetting with 5 mL differentiation medium into a 15-mL Falcon tube.
4. Centrifuge the cells at 100*g* for 5 min and remove supernatant.
5. Using a cell strainer, filter the cells suspended with differentiation medium into a 50-mL tube.
6. Incubate single-cell suspensions in a 37°C incubator with 5% CO_2 environment for 20 min (*see* **Note 5**).
7. Centrifuge the cells at 100*g* for 5 min, remove supernatant, and suspend cells with ice-cold MACS solution (10^7 cells per 50 µL) in a 1.5-mL tube.
8. Add appropriate amount of anti-Flk-1 antibodies (*see* **Note 6**) and incubate at 4°C for 15 min.
9. Add 1 mL MACS solution, centrifuge the cells at 100*g* for 3 min, remove supernatant, and suspend cells with MACS solution (10^7 cells per 80 µL).
10. Add appropriate amount of anti-PE microbeads (20 µL beads for 10^7 cells) and incubate at 4°C for 15 min.

11. Add 1 mL MACS solution, centrifuge the cells at 100*g* for 3 min, remove supernatant, and suspend cells with 500 µL MACS solution.
12. Load the cells onto the equilibrated MACS separation column on the separator. Cells labeled with microbeads are retained on the MACS column while the unlabeled cells pass through. Wash the column three times with 500 µL MACS solution.
13. Remove the column from the separator and add 3 mL MACS solution to elute the Flk-1+ cells into a 15-mL Falcon tube. Centrifuge the eluted Flk-1+ cells at 100*g* for 5 min and suspend them in differentiation medium.
14. Plate 2×10^4 Flk-1+ cells into each well of a collagen type IV-coated 24-well plate containing 1 mL differentiation medium supplemented with 30 ng/mL VEGF (*see* **Note 7**).
15. Incubate in a 37°C incubator with 5% CO_2 environment for 3–4 d (*see* **Notes 8** and **9**).

3.2. Staining of ESC-Derived Vascular Cells

1. Remove the tissue culture medium by aspiration and wash the cells once with PBS.
2. Aspirate the wash buffer and fix the cells by adding 4% PFA. Incubate for 10 min at room temperature.
3. Remove the PFA by aspiration and wash the cells twice with PBS.
4. Aspirate the last wash buffer and block the endogenous peroxidase activity by adding 0.3% H_2O_2/MeOH. Incubate for 20–30 min at room temperature.
5. Remove the H_2O_2/MeOH by aspiration and wash the cells twice with PBS.
6. Aspirate the last wash buffer and block the nonspecific antibody binding by adding 2% skim milk/PBS. Incubate for 30 min at room temperature.
7. Aspirate the blocking buffer and add the primary antibodies (1:200 diluted anti-PECAM antibody [Mec13.3] and 1:400 diluted anti-SMA antibody [1A4] in 2% skim milk/PBS) to the cells. Incubate for 2–4 h at room temperature or overnight at 4°C.
8. Remove the primary antibodies by aspiration and wash the cells three times with PBS-T.
9. Aspirate the blocking buffer and add the secondary antibodies (1:200 diluted HRP-goat anti-mouse IgG antibody and AP goat anti-rat IgG antibody in 2% skim milk/PBS) to the cells. Incubate for 2 h at room temperature.
10. Remove the secondary antibodies by aspiration and wash the cells three times with PBS-T.
11. Aspirate the last wash buffer and visualize the cells by adding four to five drops Histofine DAB solution (*see* **Note 10**). Incubate for 20–30 min (until mural cells are stained brown) at room temperature.
12. Remove the Histofine DAB solution by aspiration and wash the cells three times with PBS-T.
13. Aspirate the last wash buffer and add the AP buffer to the cells. Incubate for 10 min at room temperature.
14. Aspirate the AP buffer and visualize the cells by adding AP buffer containing NBT/BCIP (1:50 dilution). Incubate for 20–30 min (until endothelial cells are stained violet) at room temperature.
15. Remove the NBT/BCIP by aspiration and wash the cells three times with PBS-T. Photograph the stained cells using a phase contrast microscope.

3.3. Western Blotting

1. Remove the tissue culture medium by aspiration and wash the cells twice with ice-cold PBS.
2. Aspirate the wash buffer and add lysis buffer, collect lysate to a 1.5-mL tube, and incubate for 20 min on ice.
3. Centrifuge for 15 min at 20,000*g*.

4. Add sample buffer to supernatant and boil at 98°C for 3 min.
5. Run samples on an SDS polyacrylamide gel, remove the plates, and cut the gel to the desired size for transfer. Mark the gel to establish orientation.
6. Prepare one sheet of transfer membrane and 18 sheets of electrode paper to the size of the gel.
7. Wet the transfer membrane in 100% methanol and soak it in transfer buffer anode 2. Wet electrode paper by soaking in transfer buffer. Place six pieces of electrode paper in anode 1, three pieces in anode 2, and nine pieces in cathode.
8. Assemble the transfer sandwich: anode pad, then six sheets absorbent paper in anode 1, three sheets absorbent paper in anode 2, membrane, gel, nine sheets absorbent paper in cathode, and cathode pad.
9. Transfer for 60–90 min at 0.8 mA/cm^2 (area of gel).
10. After transfer, disconnect the power supply. Rinse the membrane several times with TBS-T.
11. Block the nonspecific antibody binding by adding 5% skim milk/TBS-T. Incubate for 60 min at room temperature.
12. Remove the blocking buffer and add the primary antibodies (1:1000 diluted anti-phospho-Smad2 antibody or 1:1000 diluted anti-phospho-Smad1/5 antibody in TBS-T). Incubate overnight at 4°C.
13. Wash the blot with three changes of TBS-T for 5 min each.
14. Add secondary antibody (1:10,000 diluted HRP-conjugated anti-rabbit IgG in 5% skim milk/TBS-T). Incubate for 30 min at room temperature.
15. Wash the membrane five times with TBS-T for 10 min each.
16. Incubate the membrane with freshly prepared chemiluminescence reagent for 1 min.
17. Drain the blot and remove excess chemiluminescence reagent. Place the membrane in plastic wrap to ensure a dry surface for film exposure.
18. Expose the membrane to X-ray film in a dark room for 1 min. Develop the film and determine the appropriate exposure time.

4. Notes

1. CCE cells generate Flk-1+ cells at a frequency of 30–40% of cells when differentiated.
2. FCS is a critical factor in the efficiency of the generation of Flk-1+ cells. Batch checks are strongly recommended before commencing large-scale studies of ESC in vitro vasculogenesis.
3. TGF-β3 and BMP7 have essentially same activities as TGF-β1 and BMP2/4/6, respectively.
4. Within 4 d of differentiation, the cell number increases approx 100-fold. The ratio of Flk-1+ cells increases toward d 4 during differentiation and decreases on d 5, while the cell number increases. As differentiated endothelial cells (i.e., CD31+ or VE-cadherin+ cells) start to be observed among Flk-1+ cells after 108 h, differentiation time should not exceed 108 h.
5. Some Flk-1 proteins are removed from Flk-1+ cells by treatment with trypsin/EDTA. To obtain good yield of purification of Flk-1+ cells, the cells need to be incubated to recover the Flk-1 proteins.
6. We usually add 1 μL antibodies for 10^6 cells.
7. BMP7 (500 ng/mL), TGF-β (3 ng/mL), or SB431542 (1 μ*M*) can be added to differentiation medium at this point to study their effects on endothelial differentiation by staining for vascular markers.
8. When cells are cultured for 4 d, the medium needs to be exchanged on d 2.

9. BMP7 (500 ng/mL), TGF-β (3 ng/mL), and/or SB431542 (1 μ*M*) can be treated to differentiated vascular cells for 1 h on d 3 during vascular differentiation to study their effects on phosphorylation of R-Smads by Western blot analysis.
10. Alternatively, incubate cells with 0.025% DAB/PBS-T, followed by addition of 1/10 volume 0.15% H_2O_2/PBS-T and incubation for 20–30 min at room temperature.

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The Role of the Adapter Protein SHB in Embryonic Stem Cell Differentiation Into the Pancreatic β-Cell and Endothelial Lineages

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Summary

Embryonic stem (ES) cells represent an attractive tool not only for the study of the development of various cell types but also as a potential source of cells for transplantation. Previous studies suggested a role of the signal transduction protein SRC homology 2(SH2) protein of Beta-cells (SHB) for the development of both pancreatic β-cells and blood vessels. SHB is an SH2 domain-containing adapter protein involved in the generation of signaling complexes in response to activation of a variety of receptors, several of which have been implicated in developmental processes. Moreover, microarray analysis of ES cells expressing mutant SHB has revealed decreased expression of several genes of developmental importance. Here, we present protocols that may be used for transfection of mouse ES cells and to study the differentiation of ES cell-derived embryoid bodies (EBs) into the pancreatic β-cell lineage as well as into vascular structures with special reference to the effect of SHB. Moreover, we also provide a protocol that may be used for enrichment by fluorescence-activated cell sorting of specific cell lineages in EBs.

Key Words: β-cell; CD31; development; ES cells; FACS; β-galactosidase; green fluorescent protein; insulin; pancreas; PDX-1; SHB; vasculogenesis.

1. Introduction

The insulin-producing β-cells of the pancreatic islets of Langerhans are of fundamental importance for metabolic control in mammals. In the developing embryo, both endocrine and exocrine pancreatic cells arise from precursors in the foregut endoderm. A number of transcription factors have been identified and shown to be of crucial importance during different stages of pancreatic development (for reviews, *see* **refs.** *1* and *2*). The transcription factor pancreatic-duodenal homeobox factor (PDX)-1 may be detected at day E9.5 in gut endoderm and is indispensable for pancreas morphogenesis *(1–3)*. The expression of PDX-1 requires inhibition of Sonic hedgehog (Shh) signaling,

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which may be induced by activin and fibroblast growth factor 2 derived from the notochord *(4)*. In the mature pancreas, PDX-1 expression becomes confined to the β-cells, in which it is necessary for the transcription of the insulin gene *(1)*.

The developing pancreas is subject to regulation by numerous signals derived from the extracellular environment. Thus, for example, a study described the positioning of the aorta and factors from the vascular endothelium as important for pancreas development *(3)*. Around E13.5, the mouse embryonic pancreas starts to undergo major changes, including fusion of the two pancreatic buds *(2)*. Endocrine lineage-committed progenitors expressing transcription factor neurogenin 3 (NGN3) appear in the epithelium of embryonic pancreatic ducts. NGN3 is believed to orchestrate the development of the endocrine lineage and may directly or indirectly activate several other transcription factors of the endocrine pancreatic cells *(2)*.

Embryonic stem (ES) cells represent not only an attractive tool for the study of pancreas and β-cell development but also a potential source of insulin-producing cells for transplantation to alleviate insulin deficiency in diabetes. ES cell lines may be derived from the inner cell mass of the blastocyst and maintained in vitro in an undifferentiated pluripotent state by culture on a feeder layer composed of embryonic fibroblasts (EFs) in the presence of leukemia inhibitory factor (LIF). On withdrawal of feeders and LIF, culture of ES cells in suspension results in the formation of embryoid bodies (EBs) in which derivatives of all three germ layers, including endoderm, develop *(5)*.

Differentiation of insulin-producing cells from ES cells has been described in a number of publications *(6–8)*. Culture of ES cell-derived EBs under standard conditions results in a small number of insulin-positive cells *(9,10)*. Several protocols to enhance the formation of insulin-expressing cells have been described *(11–15)*. In most cases, however, insulin-positive cells derived using protocols designed hitherto have failed to cure diabetes in mice. Presumably, the extensive knowledge about normal pancreas and β-cell development in the embryo gained during recent years may be applied to ES cells to promote the differentiation of functional insulin-producing β-cells. Furthermore, in view of the modest proportion of pancreatic precursors in differentiated EBs, methods to enrich such cells by fluorescence-activated cell sorting (FACS) may potentially allow for the subculture of cells that could be induced to undergo further differentiation into insulin-producing β-cells.

Previous studies from our lab suggest a role of the signal transduction protein SRC homology 2 (SH2) protein of Beta-cells (SHB) for the development of pancreatic β-cells *(16)*. Thus, a transgenic mouse expressing SHB under control of the insulin promoter exhibited an increased β-cell mass at birth as well as enhanced glucose tolerance and insulin secretion *(16)*. Conversely, expression of mutant SHB in ES cell-derived EBs resulted in decreased expression of both insulin and glucagon mRNA, whereas PDX-1 mRNA decreased less *(17)*. SHB is a ubiquitously expressed SH2 domain-containing adapter protein that has been shown to be involved in the generation of signaling complexes in response to activation of a variety of receptors, including the tyrosine kinase receptors fibroblast growth factor receptor 1, vascular endothelial growth factor receptor (VEGFR) 2, and platelet-derived growth factor (PDGF) receptors, all of which have been implicated in developmental processes (*17–20* and references

therein). In addition, SHB was found to operate downstream of the T-cell receptor *(21)* and nerve growth factor receptor TRKA *(22)*.

In view of its involvement in signaling downstream of VEGFR-2 and PDGF receptors, it is not surprising that SHB has been found to play a role also in blood vessel formation *(23)*. During the embryonic period, blood vessels develop from mesodermal precursors in the yolk sac (for a review, *see* **ref.** *24*). The hemangioblast represents a common progenitor for both endothelial cells and blood cells *(24)*. Also, in EBs in vitro, the development of a vascular plexus along with the expression of VEGFR-2 can be observed *(24–27)*. By d 8 in EBs, the crude vascular plexus is replaced by a finer, more differentiated one that expresses endothelial cell markers, including CD31 and VEGFR-2 *(24–27)*. The development of these plexa may be stimulated by angiogenic factors such as vascular endothelial growth factor (VEGF) and PDGF *(26)*. Blood vessel formation appears dramatically impaired in EBs expressing mutant SHB, whereas overexpression of normal SHB results in enhanced vessel formation and expression of VEGFR-2 *(28)*. Indeed, microarray analysis of ES cells expressing the mutant SHB has revealed altered expression of 128 genes, the majority of which were decreased *(17)*. Several of these have been identified as transcription factors involved in development, including retinoic acid receptors, GLI-Kruppel family member GLI 2, SRY-box containing gene 17 (Sox 17), Kruppel-like factor 7, H19 mRNA, gastrulation brain homeobox 2 (Gbx2), and others *(17)*. In addition, laminin 1 and collagen IV were decreased, which may contribute to the impaired formation of blood vessels in mutant SHB EBs *(17,27,28)*.

Here, we present protocols that may be used for transfection of mouse ES cells and to study the differentiation of ES cell-derived EBs into the pancreatic β-cell lineage as well as into vascular-forming endothelial cells with special reference to the effect of SHB. Moreover, we also provide a protocol for enrichment of specific cell lineages in EBs by FACS.

2. Materials

2.1. Tissue Culture

1. Dulbecco's modified Eagle's medium (DMEM) with Glutamax (Gibco, Invitrogen Ltd., Paisley, UK; cat. no. 61965-026).
2. Fetal bovine serum (FBS) (Gibco, cat. no. 10270-106) (*see* **Note 1**).
3. Sodium pyruvate (Gibco, cat. no. 11360-039).
4. HEPES buffer (Gibco, cat. no. 15630-056).
5. Monothioglycerol (Sigma-Aldrich, St. Louis, MO; cat. no. M-6145).
6. Benzylpenicillin-streptomycin (500X, lyophilizate, sterile) (Roche Diagnostics, GmbH, Mannheim, Germany; cat. no. 1074440): for 20 mL, dissolve the lyophilizate in 20 mL sterile distilled water and store at –20°C in 1-mL aliquots.
7. LIF 10^7 U/1 mL (ESGRO, Chemicon International Inc., Temecula, CA; cat. no. ESG1107).
8. 50-mL tubes (Sarstedt Inc., Newton, NC; cat. no. 62.547.254).
9. Calcium- and magnesium-free Hanks solution (Sigma-Aldrich, cat. no. H9394).
10. Trypsin-ethylenediaminetetraacetic acid (EDTA) (10X; Sigma-Aldrich, cat. no. T4174): to prepare 50 mL trypsin solution, combine 5 mL trypsin-EDTA with 45 mL calcium- and magnesium-free Hanks solution. Store at 4–8°C.
11. 1.5-mL Safe-lock tubes (Eppendorf AG, Hamburg, Germany; cat. no. 0030 121.848).

12. 9-cm Petri dish (Heger AS, Rjukan, Norway; cat. no. 42-00 1065).
13. Six-well, flat-bottom tissue culture plate (Sarstedt, cat. no. 83.1839).
14. 25-cm^2 tissue culture flask (Sarstedt, cat. no. 83.1810).
15. 175-cm^2 tissue culture flask (Sarstedt, cat. no. 83.1812.002).
16. Six-well, flat-bottom suspension cell tissue culture plate (Sarstedt, cat. no. 83.1839.500).
17. 24-well Cell+ tissue culture plate (Sarstedt, cat. no. 83.1836.300).
18. 96-well, flat-bottom tissue culture plate with lid (Sarstedt, cat. no. 83.1835).
19. Phosphate-buffered saline (PBS) tablets (Medicago AB, Uppsala, Sweden; cat. no. 09-9402-100): for 1 L, dissolve one tablet in 1 L distilled water and stir. Autoclave.
20. 13-mL tubes (Sarstedt, cat. no. 62.515.006).
21. Dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany; cat. no. 1.02934.100).

2.1.1. Media

1. Medium for EFs: to a 500-mL bottle of DMEM with Glutamax, add 55 mL FBS, 7.5 mL sodium pyruvate, and 1 mL benzylpenicillin-streptomycin.
2. Medium for ES cells: the medium used for expansion and maintenance of ES cells in the undifferentiated state is identical to that used for EBs (*see* **Subheading 2.1.1., item 3**), with the notable exception that LIF should be added at a final concentration of 1000 U/mL. To prepare 100 mL ES medium, add 10 µL LIF to 100 mL EB medium.
3. Medium for formation and differentiation of EBs: to a 500-mL bottle of DMEM with Glutamax, add 90 mL FBS, 7.5 mL sodium pyruvate, 15.5 mL HEPES buffer, 7.75 µL monothioglycerol, and 1 mL benzylpenicillin-streptomycin.
4. ES freezing medium: to prepare 10 mL, add 1 mL DMSO and 1.35 mL FBS to 7.65 mL ES medium.
5. 2X EF freezing medium: 6.7 mL EF medium, 5.3 mL FBS, and 3 mL DMSO.

2.1.2. General Comments and Required Equipment for Tissue Culturing

Culture ES cells and EBs under sterile conditions and in sterile culture medium. The following equipment is required:

1. Humidified cell culture incubator at 37°C and 5% CO_2.
2. Laminar flow cabinet.
3. Inverted light microscope with phase contrast objectives (×4 to ×40).
4. Tiefe 0.100-mm, 0.0025-mm^2 Bürker chamber (Assistent, Glaswarenfabrik, Karl Hecht KG, Sondheim/Rhön, Germany) with cover slip for cell counting.
5. Autoclave for sterilization of equipment and solutions.
6. 10-, 200-, and 1000-µL Biosphere filter tips (Sarstedt, cat. no. 70.1115.210, 70.760.211, and 70.762.211, respectively).
7. 5-, 10-, and 25-mL pipets (Sarstedt, cat. no. 86.1253.001, 86.1254.001, and 86.1685.001, respectively).
8. Tabletop centrifuge (Eppendorf, model no. 5415C).
9. Centrifuge (Jouan Inc., Winchester, VA; model no. C422).
10. Refrigerator (4°C) and freezers (–20, –70, and –130°C).

2.2. Electroporation of ES Cells

1. Gene Pulser II apparatus (Bio-Rad Laboratories, Hercules, CA).
2. Gene Pulser Cuvet (Bio-Rad Laboratories, cat. no. 1652088).
3. PBS (*see* **Subheading 2.1., item 19**).
4. Appropriate antibiotic for selection of stable clones.

2.3. Fluorescence-Activated Cell Sorting

1. Collagenase A from *Clostridium histolyticum* (Roche Diagnostics, cat. no. 10154121) (*see* **Note 2**).
2. Hanks balanced salt solution (Gibco, cat. no. 34020-091).
3. PBS (*see* **Subheading 2.1.**, **item 19**).
4. EDTA (Titriplex III) (Merck, cat. no. 1.08418).
5. BD Falcon cell strainer (BD Bioscience Discovery Labware, Bedford, MA; cat. no. 352340).
6. Becton Dickinson FACSCalibur instrument (BD Bioscience).
7. *CELLQuest* software (BD Bioscience, cat. no. 01-21207-02).
8. 20-L FACSFlow sheath fluid (BD Bioscience, cat. no. 342003).
9. Cytoslide (Shandon Inc., Pittsburgh, PA; cat. no. 5991051).
10. Centrifuge Cytospin 3 (Shandon).

2.4. Immunofluorescence

1. PBS (*see* **Subheading 2.1.**, **item 19**).
2. Paraformaldehyde (Merck, cat. no. 1.04005.1000): to prepare 100 mL 4% paraformaldehyde, dissolve 4 g paraformaldehyde in 100 mL PBS at 60°C while stirring. Add 1 *M* NaOH dropwise until a clear solution is obtained. Adjust to pH 7.4 using HCl.
3. Humidified chamber (*see* **Note 3**).
4. Tris-NaCl-blocking reagent (TNB) blocking buffer: in a 250-mL glass flask, combine 10 mL 1 *M* Tris-HCl at pH 7.5 with 15 mL 1 *M* NaCl and 75 mL distilled water. While stirring, slowly add 0.5 g blocking reagent provided in the NEL700 kit (*see* **Subheading 2.6.**, **item 4**). Heat gradually to 60°C while stirring to completely dissolve the reagent. Aliquot and store at –20°C (*see* **Note 4**).
5. Tris-buffered saline (TBS): to prepare 1 L TBS, dissolve 8 g NaCl, 0.2 g KCl, and 3 g Tris-base in 800 mL distilled water. Adjust to pH 7.4 using HCl. Fill up to 1 L with distilled water.
6. Triton X-100 (Sigma-Aldrich, cat. no. 9002-93-1).
7. Primary anti-PDX-1 antibody (provided by D. A. Melton, Harvard University, Boston, MA) diluted 1:100 in PBS with 0.1% Tween-20 (Sigma-Aldrich, cat. no. P-7949).
8. Primary anti-insulin antibody (Chemicon, cat. no. AB3440) ***(29)*** diluted 1:100 in TNB with 0.1% Triton.
9. Secondary antibody: Alexa Fluor 568 goat anti-rabbit immunoglobulin (Ig) G (Molecular Probes Inc., Eugene, OR; cat. no. A11011) diluted 1:1000 in TNB with 0.2% Tween.
10. Mounting medium: Fluoromount-6 (Southern Biotechnology Associates Inc., Birmingham, AL; cat. no. 0100-01).
11. Inverted fluorescence microscope (Nikon Eclipse, Nikon, Tokyo, Japan; model no. E1000) equipped with objectives (×2 to ×40) and filters for excitation at 465–495 nm and 540–580 nm and emission at 515–555 and 600–660 nm for green and red fluorescence, respectively.
12. Camera (Nikon digital camera, Nikon, model no. DXM1200) and *ACT-1*, version 2 software (Nikon).

2.5. β-Galactosidase Staining

1. PBS (*see* **Subheading 2.1.**, **item 19**).
2. Paraformaldehyde (*see* **Subheading 2.4.**, **item 2**).
3. Galactosidase β-(gal) staining set (Roche Diagnostics, cat. no. 1828673).

2.6. Immunohistochemical Staining of Vascular Endothelial Cells

1. PBS (*see* **Subheading 2.1.**, **item 19**).
2. Paraformaldehyde (*see* **Subheading 2.4.**, **item 2**).

3. Freshly prepared 3% H_2O_2 in methanol: in a 13-mL tube, combine 1 mL 30% H_2O_2 (Merck, cat. no. 1.07209.1000) with 9 mL methanol (Merck, cat. no. 1.06009.2500).
4. TSA biotin system (PerkinElmer Life Sciences Inc., Boston, MA; cat. no. NEL700).
5. TNB blocking buffer (*see* **Subheading 2.4.**, **item 4**).
6. Primary antibody: rat anti-mouse CD31 antibody (Pharmingen, BD Biosciences, cat. no. 553370).
7. Secondary antibody: biotinylated goat anti-rat IgG (Vector Laboratories Inc., Burlingame, CA; cat. no. SK-4200).
8. Tris-NaCl-Tween-20 (TNT) wash buffer: combine 10 mL 1 *M* Tris-HCl at pH 7.5 with 15 mL 1 *M* NaCl, 75 mL distilled water, and 50 µL Tween-20.
9. AEC peroxidase substrate kit (Vector Laboratories Inc., cat. no. SK-4200).

2.7. Reverse Transcriptase Polymerase Chain Reaction

1. 10- and 200-µL Biosphere filter tips (*see* **Subheading 2.1.2.**, **item 6**).
2. RNeasy Protect minikit (Qiagen, Hilden, Germany; cat. no. 74126).
3. β-Mercaptoethanol (Sigma-Aldrich, cat. no. M-6250).
4. 27-gage needle 3/4, 0.4 × 19 (BD Microlance 3, BD, Drogheda, Ireland; cat. no. 302200).
5. 1-mL sterile syringe (BD Plastipak 3, BD, cat. no. 300013).
6. RNase-free DNase set (Qiagen, cat. no. 79254).
7. QuantiTect SYBR green reverse transcriptase polymerase chain reaction (RT-PCR) kit (Qiagen, cat. no. 204243).
8. Forward and reverse primers in separate stocks diluted to 6 µ*M*.
9. 20-µL LightCycler capillaries (Roche Diagnostics, cat. no. 11909339001).
10. LightCycler centrifuge adapters in cool block (Roche Diagnostics, cat. no. 1909312).
11. LightCycler instrument (Roche Diagnostics).
12. Qiagen OneStep RT-PCR kit (Qiagen, cat. no. 210212).
13. 0.2-mL Thermo-Tube (ABgene, Surrey, UK; cat. no. AB-0337/G).
14. PCR Express (Hybaid Ltd., Ashford, Middlesex, UK) equipped with a hot lid.
15. Agarose gel electrophoresis apparatus and reagents.
16. Ultraviolet board with camera.
17. *Kodak Digital Science 1D* software for densitometric scanning of bands (Kodak, Rochester, NY).

3. Methods

3.1. Tissue Culture

3.1.1. Freezing Cells

1. When reaching approx 70% confluency, rinse growing ES cells once with calcium- and magnesium-free Hanks solution or sterile PBS.
2. To a six-well culture plate, add approx 700 µL trypsin solution to each well and incubate for 3–5 min at 37°C.
3. When cells are starting to detach, resuspend the cells gently using a pipet with a 1000-µL filter tip and transfer to an Eppendorf tube containing 500 µL ES or EB medium (to rapidly neutralize trypsin activity).
4. Pellet the cells by centrifugation at 8000*g* for 10 s (*see* **Note 5**).
5. Resuspend the cells in 1 mL freezing medium and keep on ice.
6. Aliquot the cells in up to five vials (Eppendorf or cryovials) and fill up with an appropriate volume of freezing medium.

7. Put the tubes in a polystyrene box, then immediately transfer to a –70°C freezer (*see* **Note 6**).
8. After a few days, move the box to a –130°C freezer for long-term storage.

3.1.2. Thawing Cells

1. Thaw ES cells rapidly by gentle agitation of the frozen tube in a 37°C water bath.
2. Transfer the content to a 13-mL tube containing 4 mL EB or ES medium and pellet the cells by centrifugation at 700*g* for 5 min.
3. Resuspend the cells in 1 mL ES medium and seed onto fibroblasts in ES medium on a flat-bottom tissue culture plate. Fill up with the appropriate volume of ES medium.

3.1.3. Fibroblast Feeder Layers

3.1.3.1. Method for the Isolation of Mouse EF Cells

1. Sacrifice two or three pregnant mice 14 dpc and wash the underside carefully with 70% ethanol. Using forceps and scissors, cut out each uterus and transfer to a Petri dish with sterile PBS.
2. Remove embryos from the uterus and put them in a second Petri dish with PBS. Under a microscope, decapitate the embryos, pull out internal organs (liver, lungs, heart, kidneys, and intestine), and transfer the embryos to a third Petri dish with PBS. Clear the embryos as much as possible from red blood cells.
3. Cut the embryos into very small pieces in a Petri dish containing 1 mL per embryo of trypsin solution. Incubate for 5–10 min at 37°C.
4. Resuspend repeatedly for 5 min using a 10-mL pipet followed by 5-min resuspension using a 5-mL pipet. Transfer the suspension to a 50-mL tube containing 25 mL EF medium.
5. Let debris sediment for 5 min and divide the 20-mL supernatant into two 175-cm^2 culture flasks per embryo used. Add EF medium to a final volume of 20 mL to each flask. Grow the fibroblasts until confluent (approx 7 d) at 37°C in 5% CO_2 without changing the medium.
6. Rinse twice with PBS and incubate in 3 mL/flask of trypsin solution for 5 min at 37°C. Add 5 mL EF medium to neutralize trypsin. Resuspend approx 10 times to obtain a single-cell suspension.
7. Transfer approx 90% of the suspension to one 50-mL tube per each culture flask. Leave 10% of the suspension in the flasks and fill up with 20 mL EF medium again for another 7-d culture to yield a second batch of EF cells.
8. Centrifuge the cells in the tubes for 3 min at 800*g*. Remove the supernatant and resuspend the cells of each tube in 35 mL EF medium.
9. Irradiate the cells in the tubes at 3000 rad. Centrifuge again and resuspend the cells in 15 mL per each tube of EF medium.
10. To each tube, add 15 mL cold 2X EF freezing medium. Resuspend again to mix, then aliquot the cell suspension of each tube into approx 30 sterile Eppendorf tubes; keep on ice for 10 min.
11. Put the tubes in a polystyrene box and transfer to a –70°C freezer. After a few days, move the box to a –130°C freezer for long-term storage (*see* **Note 6**).

3.1.3.2. Maintenance of EF Cells

EF cells may be stored at –130°C as described above for at least a year.

1. To each well of a six-well, flat-bottom culture plate, add 2 mL EF, EB, or ES medium.
2. Thaw an Eppendorf tube of EF cells and resuspend and divide the content to each of the six wells.
3. Culture the EF cells at 37°C in 5% CO_2. ES cells may be seeded after 3 h or up to at least 10 d later.

3.1.4. ES Cells in Culture

Mouse ES 129-R1 cells are maintained in the undifferentiated state by culture on a feeder layer composed of mouse EF cells in ES medium at 37°C in 5% CO_2.

1. Passage ES cells every 4–6 d when reaching approx 70% confluency by trypsinizing growing ES cells (*see* **Subheading 3.1.1.**, **steps 1–4**).
2. After neutralization of trypsin activity and centrifugation, resuspend the cells in 1 mL ES or EB medium and transfer 40–80 µL to a new six-well, flat-bottom tissue culture plate containing EF cells in ES medium. Keep track of the passage number.

3.1.5. Transfection of ES Cells by Electroporation

Electroporation is currently the most widely used method for transfection of ES cells. We have used electroporation to achieve stable ES cell (129-R1) clones overexpressing wild-type human SHB or SHB with a point mutation in the SH2 domain (R522K-SHB) ***(17)***. In both cases, the pCAGGS plasmid was used in which the gene of interest is inserted under control of the CMV enhancer β-globin promoter. Each of the constructs was co-transfected with pMSCVhph containing the hygromycin resistance gene, and stable clones were selected by culture in the presence of 0.2 mg/mL hygromycin B ***(17)***. The protocol here may be used for electroporation of ES cells and subsequent selection of stable clones.

3.1.5.1. Preparation of DNA

In general, linearized DNA is preferred, especially to achieve stable transfection by electroporation.

1. Linearize the plasmid using a restriction enzyme that cuts at a single site but not in the gene of interest or any important regulatory sequence of the construct.
2. Run an aliquot on an agarose gel to confirm the appearance of a single band of the expected size.
3. Preferably precipitate the DNA and wash with 70% ethanol for sterility (*see* **Note 7**).
4. If the plasmid harboring the gene of interest does not contain a selection gene, then the cells may be co-transfected with a second plasmid containing such a gene. Combine 4 µg of a plasmid containing a resistance gene with 40 µg plasmid DNA containing the gene of interest and use sterile PBS to adjust the volume to 150 µL.

3.1.5.2. Preparation of Cells and Electroporation

1. On the Gene Pulser II apparatus, connect cables and switch on the device. Use the high-capacitance interval and set value to 500 µF. Set voltage to 240 V (*see* **Note 8**).
2. When reaching approx 70% confluency, trypsinize ES cells ($1–3 \times 10^7$ cells) (*see* **Subheading 3.1.1.**, **steps 1–4**).
3. Resuspend the cells in sterile PBS to rinse. Pellet the cells again by centrifugation and resuspend in 650 µL sterile PBS and transfer to a Gene Pulser cuvet on ice.
4. Add the 150-µL sample of DNA and mix gently using a pipet. Do not introduce air bubbles. Wipe the cuvet dry with paper and place it in the holder.
5. Discharge the device by simultaneously pressing both red buttons and release as soon as the machine emits a sound. Note the pulse interval.

6. Take the cuvet out of the holder and check for small bubbles at the surface, a sign that indicates successful electroporation.
7. Immediately seed the ES cells onto feeder cells in ES medium in a tissue culture well.

3.1.5.3. Selection of Positive ES Cell Clones

Grow the cells for 48–72 h in ES medium before initiating selection. After this time period, change medium to ES medium containing the appropriate antibiotic (e.g., containing 0.2 mg/mL hygromycin B). Change medium daily. Clones may often be picked after 6 d of culture in the selective medium. If the clones are still very small after 8 d of selection, then switch to normal ES medium and grow another 2–3 d before picking.

To pick stable clones:

1. Wash cells once with calcium- and magnesium-free Hanks solution and add new Hanks to cover the growing cell clones.
2. Under an inverted microscope, pick clones using a pipet with sterile disposable tips and transfer each clone to Eppendorf tubes containing 50–100 µL trypsin solution (*see* **Note 9**).
3. Incubate for 3 min in cell incubator for trypsinization and disperse the cells by pipetting. Seed the cells of each clone onto fibroblasts in ES medium on a 96-well tissue culture plate to expand the clones.
4. When cells reach 70% confluency, trypsinize the cells again and continue expansion on a larger tissue culture plate.
5. Analyze the cell clones for expression of the transgene (e.g., by Western blot analysis or RT-PCR) and freeze aliquots of the clones in tubes marked with passage number (*see* **Note 10**).

3.1.6. Differentiation of ES Cells

3.1.6.1. EB Formation

Culture of ES cells in suspension in the absence of feeders and LIF results in the reaggregation of cells and the formation of EBs. Subsequent plating and outgrowth of EBs on attachment culture dishes leads to the development of the three germ layers: mesoderm, ectoderm, and—to a lesser extent—endoderm (from which the pancreatic β-cells emanate) *(5,7)*. To induce the formation of EBs from ES cells:

1. When approx 70% confluent, disperse growing ES cells by trypsin treatment (*see* **Subheading 3.1.1.**, **steps 1–4**). Resuspend the cells in 1 mL EB medium.
2. To remove fibroblasts, transfer the cells to a 25-cm^2 tissue culture flask and adjust the volume to 2 mL using EB medium. Incubate for 20–30 min (this time may vary between cell clones) in the cell incubator to allow fibroblasts but not ES cells to attach. After this time period, turn the flask upside down and culture for another 20–30 min to remove most remaining fibroblasts.
3. From the ES cells in suspension, take an aliquot for cell counting. Seed 10^5 cells/cm^2 ES cells in a six-well (nonattachment) culture plate for suspension cells.
4. Culture the ES cells free floating in EB medium for 4 d to induce the formation of EBs.

3.1.6.2. Differentiation of EBs

On d 4 following the start of EB formation (*see* **Note 11**), pick EBs manually under the microscope using a pipet and transfer to a 24-well (6–9 EBs/well) or a 6-well

(20–30 EBs/well) tissue culture plate, depending on the purpose (*see* **Note 12**). Culture the EBs further in EB medium for up to 21 d to allow outgrowth and differentiation. Change medium when needed (as indicated by the color of the culture medium or the stability of any added factor). In the beginning of EB culture (up to 1–1.5 wk), it may be sufficient to change the medium only every 3 d; toward the end, it may have to be changed every 1–2 d.

3.2. Analysis of Differentiation

3.2.1. FACS Analysis and Sorting of Green Fluorescent Protein-Positive Cells in EBs

FACS analysis of green fluorescent protein (GFP) in EBs expressing a GFP under control of a cell lineage-specific promoter represents a fast and convenient method, for instance, to screen for treatments that result in enhanced formation of a particular cell lineage in EBs at different stages of differentiation. Moreover, sorting by FACS of cells of a specific lineage may potentially be used to enrich cells that could be cultured and subjected to protocols aimed at further differentiation. To sort GFP-positive cells in EBs for subsequent analysis by immunofluorescence or RT-PCR:

1. Remove EB medium from EBs and rinse once in sterile PBS. To disperse the cells of the EBs, add a freshly prepared solution of collagenase (2.5 mg/mL in Hanks balanced salt solution) in a volume sufficient to cover the EBs in the well and incubate at 37°C.
2. After a 10-min incubation, gently resuspend several times using a 10-mL pipet until all the EBs detach from the bottom of the well. After another 10–15 min of incubation, resuspend several times using a pipet with a 1000-µL tip until a single-cell suspension is obtained.
3. Transfer the cells dropwise to a cell strainer in a 50-mL tube on ice. Rinse the culture well with a cold solution of 0.5 m*M* EDTA in PBS and add this dropwise to the cell strainer.
4. Centrifuge the tube at 500*g* for 5 min and carefully remove the supernatant. Add 5–10 mL EB medium and gently resuspend the cells.
5. Pellet the cells again by centrifugation and resuspend the cells gently in 1–2 or 3–6 mL (for EBs retrieved from a 24-well or a 6-well plate, respectively) of EB medium.
6. Feed the cells to the FACS instrument using forward scatter on the *x*-axis and FL1-H (for green fluorescence) on the *y*-axis as parameters.
7. To sort GFP-positive cells, set the sorting gate to include a low percentage (e.g., 0.5–1.5%) of total cells with the highest green fluorescence. Collect the sorted cells in 50-mL tubes containing 5 mL FBS. Generally, a total of 2–4 $\times 10^4$ sorted cells is enough to perform immunofluorescence or RT-PCR analysis. Remaining nonsorted cells may be used as control.
8. Pellet the cells in the 50-mL tubes by centrifugation at 800*g* for 3 min and carefully remove most of the supernatant, leaving merely a few hundred microliters in which the cells are gently resuspended.
9. For immunofluorescence, apply the cells to slides (Cytoslide) by centrifugation at 250*g* for 5 min using the Cytospin 3 centrifuge.
10. For RT-PCR, transfer the cells to Eppendorf tubes and centrifuge for 15 s at 8000*g*. Remove all of the supernatant and proceed with extraction of RNA and RT-PCR.

For a simple but fairly accurate analysis of the percentage of GFP-positive cells, use EBs derived from ES cells that are not transfected with the GFP construct to set the gate

to include a low percentage (e.g., 0.1–0.5%) of cells with the highest FL1-H fluorescence as background. Using the same gate, determine the percentage of cells within the gate from EB cells that do express the GFP construct and calculate the number of GFP-positive cells by subtracting the background value (*see* **Fig. 1**).

3.2.2. Immunofluorescence

The following protocol for immunofluorescence may be used to study the expression of PDX-1 and insulin in cells retrieved from EBs:

1. To cells on slides, apply fixative (4% paraformaldehyde) for 20 min at room temperature followed by overnight incubation in 70% ethanol at 4–8°C.
2. Rinse the slides with PBS (in which they may also be stored at 4–8°C). Put the slides in a humidified box and perform blocking in TNB with 0.1% Triton for 1 h at room temperature.
3. Incubate the slides in an appropriate dilution of primary antibody at 4°C overnight.
4. Rinse the slides four times for 15 min in TBS with 0.2% Tween followed by 1 h incubation at room temperature with an appropriate fluorescent secondary antibody.
5. After washing again (*see* **Subheading 3.2.2., Step 4**), mount the cells on the slides in mounting medium and apply cover slips.
6. Examine the slides using a fluorescence microscope.

3.2.3. β-Gal Staining for PDX-1/lacZ in EBs

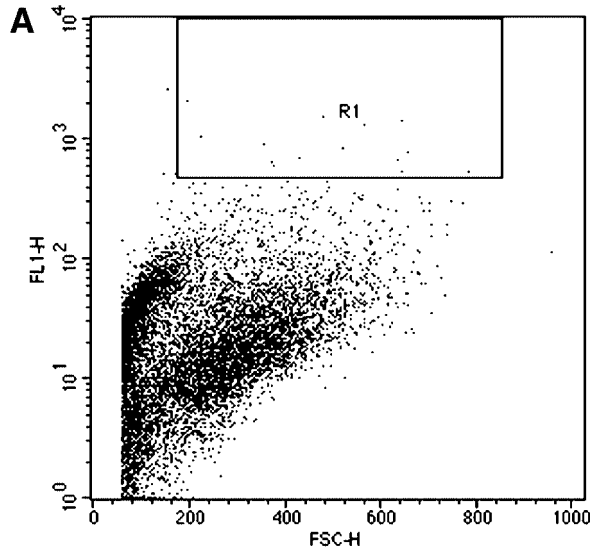
The ES 129-R1 line in use in our lab is equipped with a PDX-1/lacZ knockin ***(30)***. This provides a convenient possibility to monitor the number of PDX-1-positive cells in differentiating EBs (*see* **Fig. 2**). To stain for β-gal activity in EBs:

1. Rinse EBs growing on attachment 24-well dishes twice in PBS and incubate in fixative (4% paraformaldehyde) at 4°C for 45 min followed by rinsing twice in PBS.
2. Meanwhile, prepare the staining solution by thoroughly mixing 1 part of the X-gal solution with 19 parts of the iron solution provided in the β-gal staining set (*see* **Note 13**).
3. Apply a volume sufficient to cover the EBs in wells and incubate at 37°C for 0.5–3 h.
4. Under an inverted light microscope without phase contrast, examine the EBs at various time points for the development of blue-green staining of cells and extend the incubation time until it develops. Rinse the wells with PBS.

3.2.4. Immunhistochemical Staining for Vascular Structures in EBs

As mentioned in the introduction, the SHB adapter protein has been shown to be involved not only in β-cell development ***(16)*** but also in blood vessel formation ***(23,28)***. Moreover, a study showed an important contribution of factors derived from the vascular endothelium for the development of the pancreas ***(3)***. We have used immunohistochemical staining for the endothelial cell marker CD31 to study the occurrence of vascular structures in EBs (*see* **Fig. 3**). The following protocol may be used to reveal the presence of vascular structures in EBs:

1. Rinse EBs growing on attachment 24-well dishes twice in PBS and incubate in fixative (4% paraformaldehyde) at 4°C for 45 min followed by rinsing twice with PBS.
2. Incubate the EBs in the wells in 3% H_2O_2 in methanol for 10 min at room temperature and wash three times for 5 min each with PBS.
3. Block unspecific binding by incubation in TNB buffer for 1 h at room temperature.

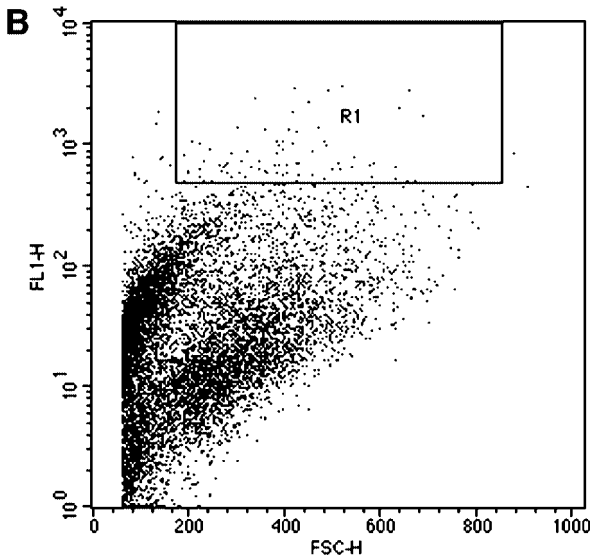


Gate Statistics

Gated Events: 10000 Total Events: 10000

X Parameter: FSC-H (Linear) Y Parameter: FL1-H (Log)

Gate	Events	% Gated	% Total
G1	14	0.14	0.14



Gate Statistics

Gated Events: 10000 Total Events: 10000

X Parameter: FSC-H (Linear) Y Parameter: FL1-H (Log)

Gate	Events	% Gated	% Total
G1	78	0.78	0.78

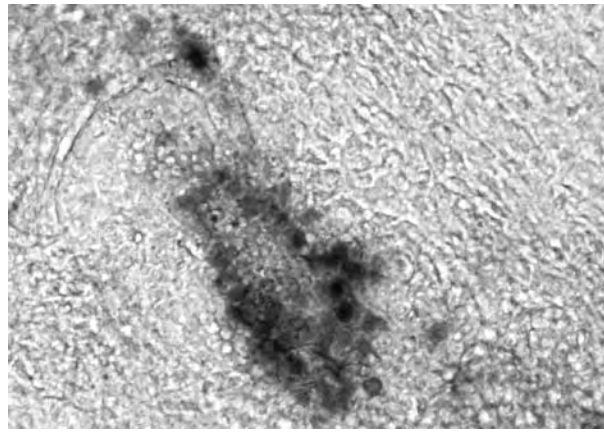


Fig. 2. β-Galactosidase staining of 21-d-old embryoid bodies derived from embryonic stem cells equipped with the PDX-1/lacZ knockin. Blue-green PDX-1/lacZ-positive cells, preferentially in groups, appear dark in the figure.

4. Incubate with rat anti-mouse CD31 antibody diluted 1:8000 in TNB for 1 h at room temperature.
5. After washing three times for 5 min each in PBS, incubate in secondary antibody of biotinylated goat antirat IgG diluted 1:300 in TNB for 30 min at room temperature. This is followed by washing three times for 5 min each with PBS.
6. Incubate in streptavidin horse radish peroxidase diluted 1:200 in TNB for 30 min at room temperature and wash again three times for 5 min each with PBS.
7. Incubate in biotinyl tyramide diluted 1:50 in 50% amplification buffer and 50% TNT buffer for 8 min at room temperature and wash again three times for 5 min each with PBS.
8. Incubate in the streptavidin horse radish peroxidase/TNB solution for another 30 min and wash again in the same manner.
9. Combine 15 µL of each of the three components provided in the AEC Peroxidase Substrate kit with 1 mL of distilled water and mix. Incubate for 15 min at room temperature and then wash again with PBS.
10. Examine the EBs under the light microscope for the presence of red-staining vascular structures.

3.2.5. Reverse Transcriptase Polymerase Chain Reaction

The expression of some of the transcripts that characterize the pancreas and β-cell lineage may be successfully detected in differentiating EBs (*see* **Fig. 4**). Primers have been established for the detection of mRNA for PDX-1 and insulin as well as human

Fig. 1. Fluorescence-activated cell sorting analysis of green fluorescent protein (GFP) expression (FL1-H) in dispersed 21-d-old embryoid bodies (EBs) derived from (**A**) control embryonic stem (ES) cells or (**B**) ES cells transfected with GFP under a cell lineage-specific promoter. The R1 gate was set to include 0.14% of total cells in **A** with the highest fluorescence as background. (**B**) The same gate as used in **A** contains 0.78% of total cells, suggesting that the proportion of the specific cell lineage in EBs in this experiment is 0.64%.

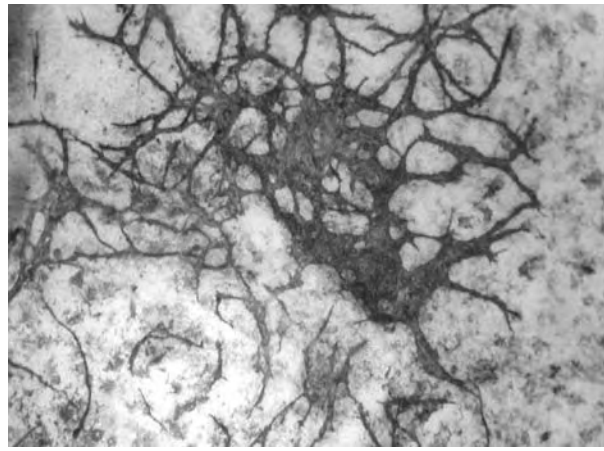


Fig. 3. Immunohistochemical staining of vascular structures in embryoid bodies (EBs). EBs were cultured for 21 d, followed by staining for the endothelial cell marker CD31.

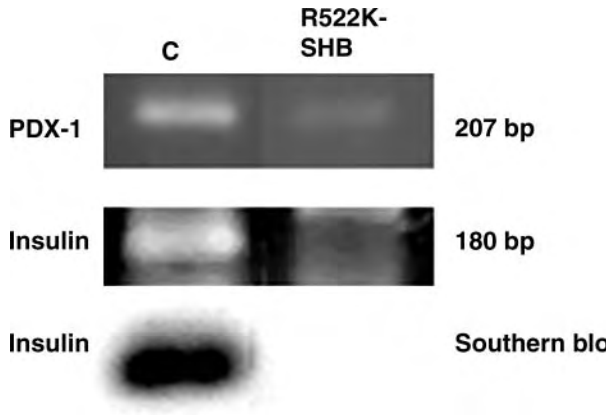


Fig. 4. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of the expression of PDX-1 (sample dilution 1:10, 35 cycles) and insulin (sample dilution 1:10, 38 cycles) mRNA in embryoid bodies (EBs) derived from control embryonic stem (ES) cells (C) or ES cells expressing mutated inactive SHB (R522K-SHB). EBs were cultured for 21 d followed by one-step conventional RT-PCR analysis after normalization of RNA levels according to the expression of a housekeeping gene. The expression of insulin mRNA was further confirmed by Southern blot analysis with hybridization to a ^{32}P-labeled insulin cDNA probe.

and mouse SHB in differentiating EBs (*see* **Table 1**). An accurate interpretation of RT-PCR requires that the level of expression of a certain mRNA in each sample is normalized to that of a gene that is assumed to be expressed at equal levels regardless of any experimental treatment of the cells. In general, a housekeeping gene such as β-actin or glyceraldehyde-3-phosphate dehydrogenase is used, but in our hands, normalization to the expression of glucose-6-phosphate dehydrogenase mRNA works well ***(31)***. When

Table 1
Primer Sequences

Gene	Forward primer	Reverse primer	Annealing temp.	Product size
mG6PDH	5′-ATT GAC CAC TAC CTG GGC AA-3′	5′-GAG ATA CAC TTC AAC ACT TTG ACC T-3′	60°C	293 bp
mPDX1	5′-ATG AAA TCC ACC AAA GCT C-3′	5′-GAT GTG TCT CTC GGT CAA GT-3′	55°C	207 bp
mInsulin[a]	5′-GGC TCT CTA CCT GGT GTG T-3′	5′-TGC AGC ACT GAT CTA CAA TG-3′	58°C	180 bp
mSHB	5′-ACA TGA AAC TGG CCA AGA CC-3′	5′-TTT TCA CAG AGT CCG CAC AG-3′	50°C	167 bp
hSHB	5′-GAT CCC TTT GAT GCC AAG AA-3′	5′-CTC TCC GAG TCC GAG TCA AC-3′	50°C	200 bp

[a]Primers are not appropriate for real-time RT-PCR using the nonspecific SYBR green.

designing primers for RT-PCR, it is preferred that half of one primer hybridizes to the 3′ end of an exon, and the other half hybridizes to the 5′ end of the adjacent exon to avoid amplification of genomic DNA. If this is not possible, then a control PCR reaction excluding RT should be carried out to check for the presence of contaminating genomic DNA (*see* **Note 14**).

3.2.5.1. Extraction of Total RNA and Removal of Genomic DNA

The following protocol has been derived mostly from the instructions provided in the Qiagen RNeasy Protect Minikit and the RNase-free DNase set. The protocol may be used for isolation of total RNA and removal of genomic DNA from up to 5×10^6 cells.

1. Combine 350 µL/sample RLT buffer with 1% (v/v) β-mercaptoethanol (*see* **Note 15**). Dissolve the lyophilized DNase in 550 µL of the provided RNase-free water. Aliquot and store at –20°C the DNase that is not used immediately. Combine 10 µL/sample DNase stock with 70 µL/sample RDD buffer and mix gently.
2. Rinse EBs growing in wells once with PBS and harvest into Eppendorf tubes by trypsinization (*see* **Subheading 3.1.1., steps 1–4**) or by using a cell scraper. Keep on ice. Spin down the cells and remove all medium. Wash once in cold PBS. Spin down the cells again and remove all PBS.
3. Add 350 µL RLT buffer (with β-mercaptoethanol) and vortex the sample thoroughly at room temperature. Pass the sample at least five times through a 27-gage 3/4 needle using a 1-mL syringe to homogenize (*see* **Note 16**).
4. Add 350 µL 70% ethanol and mix well by pipetting.
5. Transfer the sample to a minispin column in a 2-mL tube. Spin for 15 s at 8000*g* or higher. Discard the flow-through.
6. Add 350 µL RW1 buffer. Spin for 15 s at 8000*g* or higher. Discard the flow-through.

7. Add 80 µL DNAse/RDD solution and incubate at room temperature for 15 min.
8. Add 350 µL RW1 buffer. Spin for 15 s at 8000*g* or higher. Discard the flow-through.
9. Put the column in a new 2-mL tube. Add 500 µL RPE buffer. Spin for 15 s at 8000*g* or higher. Discard the flow-through.
10. Repeat **step 9** using the same tube but spin for 2 min at maximum speed.
11. Put the spin column in a new 1.5-mL tube. Add 30–50 µL of RNase-free water onto the column. Spin for 1 min at 8000*g* or higher to elute.
12. If the yield is less than 30 µg, then **step 11** may be repeated with another 30–50 µL RNase-free water. Freeze the RNA sample in aliquots at –70°C.

3.2.5.2. One-Step Real-Time RT-PCR

Real-time RT-PCR using Qiagen QuantiTect SYBR green RT-PCR kit may be performed for specific PCR products of small size (preferably 100–150 bp). It is not appropriate for PCR reactions that result in more than one single product. The following protocol refers to real-time RT-PCR using the LightCycler instrument:

1. With reference to the size of the PCR product and the T_m of the primers, which may be calculated as $T_m = 2°C \times (\text{Number of [A + T]}) + 4°C \times (\text{Number of [C + G]})$, program the LightCycler instrument as follows:

Cycles	Analysis	Temperature	Time	Ramp	Acquisition	Comment
1	None	50°C	20 min	20°C/s	None	RT
	None	95°C	15 min	20°C/s	None	Activation
35–55	Quant.	94°C	15 s	20°C/s	None	Denaturation
	Quant.	5–8°C below T_m	25 s	20°C/s	None	Annealing (*see* **Note 17**)
	Quant.	72°C	5 s/100 bp (at least 10 s)	2°C/s	Single	Extension
1	Melting	95°C	0 s	20°C/s	None	Melting curve
		T_m	20 s	20°C/s	None	
		95°C	0 s	0.1°C/s	Cont.	
1	None	40°C	30 s	20°C/s	None	Cooling

2. Thaw and vortex gently the RNA samples, primers, and the SYBR master mix and keep on ice. Also, thaw RNase-free water for use both in the mix discussed here and for a negative control in the PCR reaction. In a clean, sterile Eppendorf tube, mix the following components at the indicated volumes multiplied by the number of samples, including the negative control: 0.7 µL RNase-free water; 5 µL 2X SYBR master mix; 1.6 µL of each primer (final concentration 1 µ*M*); and 0.1 µL RT enzyme mix.
3. To precooled LightCycler capillaries in a cool block, add 1 µL (1 pg to 1 µg) RNA sample (or 1 µL RNase-free water as a negative control) and 9 µL prepared mix. Apply lids to the capillaries and spin at 700*g* for 15 s. Put the capillaries in the carousel of the LightCycler and run the RT-PCR.
4. Determine the level of relative expression by subtracting the crossing point as analyzed using the LightCycler software with the crossing point for the expression of the housekeeping gene for each sample.

3.2.5.3. One-Step RT-PCR

The Qiagen OneStep RT-PCR kit may be used for conventional RT-PCR instead of real-time PCR using the nonspecific QuantiTect SYBR green RT-PCR when real-time PCR is not applicable, for instance, because of the occurrence of more than one PCR product. However, as opposed to real-time RT-PCR, an accurate interpretation of results using this method requires the analysis of consecutive dilutions of each sample to determine the optimal number of cycles that results in PCR product within the exponential phase of amplification.

1. With reference to the T_m of the primers (*see* **Subheading 3.2.5.2.**, **step 1**), program the PCR machine as follows:

Stage	Step	Temperature	Time	Cycles	Comment
1	1	50°C	30 min		RT
	2	95°C	15 min		Activation
	3	0	0	1	
2	1	94°C	1 min		Denaturation
	2	5–8°C below T_m	1 min		Annealing (*see* **Note 17**)
	3	72°C	1 min		Extension
	4	0	0	25–40	(*see* **step 4**)
3	1	72°C	10 min		Extension
	2	0	0	1	

2. Thaw and vortex gently the RNA samples and keep on ice. Regarding the expression of the housekeeping gene, normalize the concentration of RNA in the samples by dilution in RNase-free water. Also use RNase-free water for a negative control in the PCR reaction and for preparation, for example, of two consecutive 10-fold dilutions (i.e., 1:10 to 1:100) of each normalized sample. Thaw and vortex primers, 5X buffer, and dNTP. In a clean, sterile Eppendorf tube, mix thoroughly (by repeated pipetting) the following components at the indicated volumes multiplied by the number of samples, including the dilutions and the negative control (*see* **Note 18**): 4.2 µL RNase-free water; 2 µL 5X buffer; 1 µL of each primer (final concentration 0.6 µ*M*); 0.4 µL dNTP; and 0.4 µL enzyme.
3. To PCR tubes on ice, add 1 µL RNA sample and 9 µL prepared mix and vortex gently. Put the tubes in the PCR machine at 50°C and run the RT-PCR.
4. Analyze the PCR product by agarose gel electrophoresis and densitometric scanning of bands of the expected size. Unless the dilution (e.g., 1:1 to 1:100) of the specific mRNA in each sample is reflected by a corresponding difference in band intensity, the RT-PCR must be repeated using an adjusted number of cycles to ensure that the PCR product is analyzed at the exponential phase of amplification.

4. Notes

1. Because the quality of the FBS may vary between batches, it is strongly recommended that each new batch is tested for its ability to maintain and propagate ES cells in the undifferentiated state. Preferably, thaw FBS at 4–8°C. We do not heat inactivate this FBS for use in ES, EF, or EB medium.

2. Store collagenase A at 2–8°C and handle the powder in a flow hood.
3. This can be a flat box containing pieces of dishcloth soaked in tap water.
4. Do not use TNB buffer that has been stored at room temperature for more than 24 h.
5. Although this protocol works well, a less-harsh procedure is to pellet the cells in a larger volume (e.g., 4 mL) ES or EB medium in a 13- or 50-mL tube and centrifuge at 700*g* for 5 min.
6. The use of an isolating polystyrene box permits slower freezing of cells.
7. To precipitate DNA, determine the volume of the solution and add 0.1 vol of 3 *M* NaAc at pH 5.2 and mix. Then, add 2–2.5 vol of ice-cold ethanol, mix again, and incubate for 20 min on ice. Centrifuge at 12,000*g* for 10 min at 0°C. Gently remove the supernatant using a pipet without disturbing the pellet (which may not be visible). Wash the pellet with 70% ethanol and centrifuge again at 12,000*g* for 5 min at 4°C. Carefully remove the supernatant and store the tube open at room temperature until all fluid has evaporated. Dissolve the pellet in an appropriate volume of sterile Tris-EDTA at pH 7.6–8.0.
8. Changing these parameters to 3 μF and 0.8 kV, respectively, in some instances may yield superior results.
9. Make sure to change tips between clones to avoid contamination.
10. As with nontransfected cell lines, it is advisable to store several stocks of low passage number of each clone.
11. Some authors maintain EBs in suspension for longer than 4 d before plating on adherent culture dishes. Although we have also observed successful plating and outgrowth of EBs after up to at least 6 d of culture in suspension, we have not determined the effect of extended suspension culture on the differentiation of EBs into the pancreatic lineage or vascular structures.
12. A 24-well plate will generate enough cells to allow analysis by FACS or RT-PCR as well as staining for PDX-1/lacZ (β-gal) or vascular structures in EBs; sorting by FACS requires at least an amount of cells generated by culture on a 6-well plate.
13. It is important to thaw (in a 37°C water bath) and vortex the X-gal solution until a clear solution is obtained.
14. For both extraction of RNA and RT-PCR, it is crucial to use clean gloves and RNase-free solutions and instruments. Preferably, devote a separate and clean area in the lab for setting up PCR reactions; especially, avoid handling of PCR product within or close to this area.
15. The RPE buffer should be dissolved the first time the kit is used by adding 4 vol of ethanol.
16. For homogenization, a needle with larger diameter (e.g., a 20-gage needle) or a pipet may be used at first if the EBs are very large or viscous.
17. The annealing time and temperature may be optimized experimentally.
18. The exchange of 2 μL H_2O per reaction for 2 μL 5X Q-Solution provided in the Qiagen OneStep RT-PCR kit in some cases may yield better results. The concentration of $MgCl_2$ in the PCR reaction may also be optimized in 0.5-m*M* steps from 2.5 m*M* (initial mix) up to 4 m*M*.

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In Vitro Differentiation of Embryonic Stem Cells Into the Pancreatic Lineage

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Summary

Embryonic stem (ES) cells represent a potential source of transplantable cells for the treatment of diabetes because of their almost unlimited proliferation capacity and the potential to differentiate into insulin-producing cells. The differentiation conditions significantly affect the development of pancreatic cells from ES cells in vitro. Here, we describe an efficient strategy for the in vitro generation of insulin-producing cells. The protocol includes the spontaneous generation of multilineage progenitor cells and their differentiation induction by growth and extracellular matrix factors into C-peptide/insulin-positive islet-like clusters. The differentiated cells release insulin in response to high glucose concentrations. No specific selection for nestin-positive cells is performed at any time of ES cell differentiation.

Key Words: C-peptide; insulin-producing cells; mouse embryonic stem cells; pancreatic differentiation.

1. Introduction

Pluripotent embryonic stem (ES) cells have the potential to self-renew and to differentiate into virtually any cell type of somatic and germ cell lineages, including endoderm representing exo- and endocrine cells of the pancreas (for review, *see* **refs.** *1* and *2*). Mouse ES cells are the most efficient experimental system with respect to ES cell generation, long-term cultivation, and multilineage differentiation. The spontaneous differentiation of mouse ES cells without the application of specific differentiation factors results in a heterogeneous progeny and a low yield of endocrine pancreatic cells *(3)*. However, the application of specific growth and extracellular matrix factors *(4,5)* or transgene expression *(6–8)* significantly enhanced the number of differentiated cell types and resulted in the generation of functional pancreatic cells.

At present, the lack of methods to enrich pancreatic progenitor cells in vitro is a major obstacle for the generation of selected and pure populations of pancreatic cells at high efficiency. So far, pancreatic progenitor cells are not clearly defined. Two cell

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types expressing the intermediate filament proteins nestin and cytokeratin 19, respectively, have been proposed as potential pancreatic progenitors. In vivo, nestin-positive cells were detected transiently at d 10.5 of embryonic development *(9)*, and nestin-expressing cells were found after cultivation of adult islets in vitro *(10,11)*. In addition, cells residing in the pancreatic ductal epithelium expressing cytokeratin 19 have been proposed as pancreatic stem or progenitor cells in vivo *(12)*.

Here, we describe the differentiation of mouse ES cells into the pancreatic lineage. The protocol follows the strategy: (1) to generate a pool of multilineage progenitor cells, including endodermal precursors by spontaneous differentiation of ES cells; and (2) to direct the differentiation into the pancreatic lineage by the application of specific differentiation and extracellular matrix factors. In addition, constitutive expression of the pancreatic developmental control gene *Pax4* in ES cells is applied to increase the number of functional islet-like cells *(7)*. The protocol is suitable to characterize the efficiency of ES cells to develop in vitro into insulin-producing cells. The methods include reverse transcriptase polymerase chain reaction (RT-PCR) analysis for the determination of pancreas-specific transcripts, immunofluorescence for the detection of pancreas-specific proteins, and enzyme-linked immunosorbent assay (ELISA) for the analysis of insulin release.

2. Materials

2.1. ES Cell Culture (see Note 1)

1. Phosphate-buffered saline (PBS): 10 g NaCl, 0.25 g KCl, 1.44 g Na_2HPO_4, 0.25 g $KH_2PO_4.2H_2O$; dissolve in 1 L tridistilled water and sterilize through a 0.22-µm filter.
2. Dulbecco's modified Eagle's medium (DMEM): dissolve 668.8 g DMEM (4.5 g/L glucose; Invitrogen, Karlsruhe, Germany; cat. no. 52100-047) into 50 L distilled water; adjust to pH 7.2 by 5.6% $NaHCO_3$ and filter-sterilize.
3. 10 m*M* β-mercaptoethanol: dissolve 70 µL β-mercaptoethanol (Serva, Heidelberg, Germany; cat. no. 28625) in 100 mL PBS; filter-sterilize.
4. ES cell culture medium; DMEM, supplemented by additives: 10 mL 100X L-glutamine (Invitrogen, cat. no. 25030-024), 10 mL 100X nonessential amino acids (Invitrogen, cat. no. 11140-035), 10 mL 10 m*M* β-mercaptoethanol, 10 mL 100X streptomycin-penicillin (Invitrogen, cat. no. 15070-063), 150 mL fetal calf serum (FCS) (Invitrogen, cat. no. 10207-106), and 10 µg leukemia inhibitory factor (LIF) prepared from LIF expression vectors (*see* **refs.** *13* and *14*) or from commercial sources (Chemicon, Temecula, CA; cat. no. LIF3010). Bring to a final volume of 1 L with DMEM and mix.
5. Freshly prepared and inactivated mouse embryonic fibroblasts (MEFs; feeder layer) in gelatinized 60-mm culture dishes (for preparation, *see* **ref.** *14*).
6. Trypsin-ethylenediaminetetraacetic acid (EDTA) (1:1) solution: prepare 0.2% (w/v) trypsin (Serva, cat. no. 37290) in PBS and 0.02% (w/v) EDTA (Sigma, Taufkirchen, Germany; cat. no. E-6758) in PBS. Sterilize through a 0.22-µm filter. Mix trypsin and EDTA solutions 1:1. Prepare fresh every week.

2.2. Generation of ES Cell-Derived Multilineage Progenitor Cells (see Note 1)

1. Iscove's modified of Dulbecco's medium (IMDM): dissolve 176.6 g IMDM (Invitrogen, cat. no. 42200-030) into 10 L distilled water and filter-sterilize.
2. 150 m*M* monothioglycerol: dissolve 13 µL monothioglycerol (3-mercapto-1,2-propanediol; Sigma, cat. no. M-6145) into 1 mL IMDM and filter-sterilize. Prepare fresh every time.

3. Differentiation medium I: IMDM supplemented with additives: 10 mL 100X L-glutamine (Invitrogen, cat. no. 25030-024), 10 mL 100X nonessential amino acids (Invitrogen, cat. no. 11140-035), 3 mL 150 m*M* monothioglycerol, 10 mL 100X streptomycin-penicillin (Invitrogen, cat. no. 15070-063), and 200 mL FCS (Invitrogen, cat. no. 10207-106). Bring to a final volume of 1 L with IMDM.
4. 0.1% gelatin: 0.1% (w/v) gelatin (Fluka, Seelze, Germany; cat. no. 48720) in distilled water; sterilize by autoclaving.

2.3. Induction of Pancreatic Differentiation (see Note 1)

1. 10 m*M* sodium borate buffer: dissolve 61.8 g H_3BO_3 in 100 mL distilled water. Adjust to pH 8.4 with NaOH.
2. Poly-L-ornithine solution: 0.1 mg/mL poly-L-ornithine (Sigma, cat. no. P-2533) in sodium borate buffer. Sterilize through a 0.22-µm filter.
3. PBS (*see* **Subheading 2.1.**, **item 1**).
4. Laminin solution: 1 µg/mL laminin (Sigma, cat. no. L-2020) in PBS.
5. Trypsin-EDTA (1:1) solution (*see* **Subheading 2.1.**, **item 6**).
6. DMEM/F12 medium: dissolve 599.8 g DMEM/F12 (4.5 g/L glucose; Invitrogen, cat. no. 32500-043) in 50 L distilled water and sterilize through a 0.22-µm filter.
7. Progesterone (1 m*M*): dissolve 59.5 mg progesterone (Sigma, cat. no. P-7556) in 10 mL PBS and filter-sterilize. Final concentration is 20 n*M*.
8. Putrescine (20 m*M*): dissolve 32.2 mg putrescine (Sigma, cat. no. P-5780) in 10 mL PBS and filter-sterilize. Final concentration is 100 µ*M*.
9. Insulin (1 mg/mL): dissolve 100 mg insulin (Sigma, cat. no. I-1882) in 100 mL PBS and filter-sterilize. Final concentration is 25 µg/mL.
10. Sodium selenite (1 m*M*): dissolve 17.3 mg sodium selenite (Sigma, cat. no. S-5261) in 10 mL PBS and filter-sterilize. Final concentration is 30 n*M*.
11. Transferrin (4 mg/mL): dissolve 100 mg transferrin (Sigma, cat. no. T-1147) in 25 mL distilled water and filter-sterilize. Final concentration is 50 µg/mL.
12. Laminin (1 mg/mL): dissolve 10 mg laminin (Sigma, cat. no. L-2020) in 10 mL PBS and filter-sterilize. Final concentration is 1 µg/mL.
13. Nicotinamide (5 *M*): dissolve 30.5 g nicotinamide (Sigma, cat. no. N-3376) in 50 mL distilled water and filter-sterilize. Final concentration is 10 m*M*.
14. Pancreatic differentiation medium: contains 20 µL 1 m*M* progesterone, 5 mL 20 m*M* putrescine, 1 mL 1 mg/mL laminin, 2 mL 5 *M* nicotinamide, 25 mL 1 mg/mL insulin, 30 µL 1 m*M* sodium selenite, 12.5 mL 4 mg/mL transferrin, 10 mL 100X B27 media supplement (Invitrogen, cat. no. 17504-044), 10 mL 100X streptomycin-penicillin (Invitrogen, cat. no. 15070-063), bring to a final volume of 1 L with DMEM/F12 and mix.
15. Pancreatic differentiation medium supplemented with 10% FCS: add 10 mL FCS (Invitrogen, cat. no. 10207-106) to 90 mL pancreatic differentiation medium and mix.

2.4. RT-PCR (see Note 2)

1. PBS (*see* **Subheading 2.1.**, **item 1**).
2. Diethyl pyrocarbonate-treated water (DEPC-H_2O): add 1 mL DEPC (Invitrogen, cat. no. 10977-015) to 1 L Milli-Q water and stir overnight. DEPC is inactivated by heating to 100°C for 15 min or autoclaving for 15 min.
3. RNA lysis buffer: add 23.6 g of guanidinium thiocyanate to 5 mL 250 m*M* Na-citrate at pH 7.0, 2.5 mL 10% Sarcosyl and add DEPC-H_2O to a total volume of 49.5 mL; mix carefully. Make fresh at monthly intervals. Add 1% β-mercaptoethanol before use.

4. 2 *M* Na-acetate at pH 4.0: dissolve 27.2 g Na-acetate × $3H_2O$ in 0.1% DEPC-H_2O, adjust to pH 4.0 with glacial acetic acid, and adjust to 100 mL with DEPC-H_2O. Treat the buffer with 0.1% DEPC-H_2O at 37°C for at least 1 h and heat to 100°C or autoclave for 15 min.
5. Acidic phenol: phenol is saturated with DEPC-H_2O instead of Tris-HCl. The saturated acidic phenol contains 0.1% hydroxyquinoline (antioxidant, partial inhibitor of RNase, and a weak chelator of metal ions; its yellow color provides a convenient way to identify the organic phase). Store at 4°C for up to 2 mo.
6. Chloroform: isoamylalcohol (24:1).
7. Isopropanol.
8. 75% ethanol: prepare in DEPC-H_2O.
9. RevertAis M-MuLV RT: add 200 U/µL with 5X reaction buffer containing 250 m*M* Tris-HCl at pH 8.3, 250 m*M* KCl, 20 m*M* $MgCl_2$, and 50 m*M* dithiothreitol (Fermentas, St. Leon-Rot, Germany; cat. no. EP0441).
10. RNase inhibitor (Fermentas, cat. no. EO0312): 40 U/µL.
11. 50 µ*M* Oligo $d(T)_{18}$ in 10 m*M* Tris-HCl at pH 8.3 (Fermentas, cat. no. S00132).
12. 100 m*M* dNTP mix: dNTP (dGTP, dATP, dCTP, dTTP; Fermentas, cat. no. R0181). 10 m*M* dNTP is prepared fresh before use.
13. Recombinant *Taq* DNA polymerase 5 U/µL with 10X PCR buffer: 750 m*M* Tris-HCl at pH 8.8, 200 m*M* $(NH_4)_2SO_4$, 0.1% Tween-20, and 25 m*M* $MgCl_2$ (Fermentas, cat. no. EP0402).
14. PCR primer pairs: dilute synthetic oligonucleotides to 10 m*M* with DEPC-H_2O and freeze at –20°C (for sequences suitable to detect expression of pancreas-specific genes; *see* **Table 1**).
15. 10X Loening solution: dissolve 10.9 g Tris-HCl, 10.4 g NaH_2PO_4 1 · H_2O, and 0.93 g EDTA in 50 mL distilled water, mix well, adjust to pH 7.7 with HCl, and autoclave.
16. Loading buffer: 10 mL 10X Loening solution, 30 mL glycerine, 10 mL 10% sodium dodecyl sulfate, 20 mL 10% *N*-lauroyl-sarcosine, and 100 mg bromcresol green (Sigma, cat. no. B-1256); add 30 mL Milli-Q water.
17. 5X TBE: dissolve 54 g Tris-base and 27.5 g boric acid in Milli-Q water, add 20 mL 0.5 *M* EDTA at pH 8.0, and adjust to 1 L with Milli-Q water.
18. Ethidium bromide aqueous solution (Serva, cat. no. 21251): 1% w/v = 10 mg/mL. For agarose gels, melt electrophoresis-grade agarose (Invitrogen, cat. no. 15510-027) in 1X TBE by gentle boiling in a microwave oven. Cool to below 60°C and pour into an agarose gel mold. Run small gels at around 80–100 V by using bromcresol green in the stop mix as an indicator of migration (*see* **Note 2**).
19. Gene Ruler 1-kb DNA ladder (Fermentas, cat. no. SM0318).

2.5. Immunohistochemical Analysis

1. PBS (*see* **Subheading 2.1., item 1**).
2. 4% Paraformaldehyde (PFA) (Fluka, cat. no. 76240): dissolve 4 g PFA in PBS and adjust to 100 mL with PBS; heat the mixture to 95°C, stir until the solution becomes clear, and cool to room temperature (*see* **Note 3**).
3. Methanol:acetone (7:3) fixative: precool to –20°C.
4. 10% goat serum (Invitrogen, cat. no. 16210-064) or 1% bovine serum albumin (BSA) in PBS for blocking unspecific binding of antibodies.
5. Hoechst 33342 (*bis*-benzimide, 5 µg/mL in PBS; Sigma, cat. no. B-2261).
6. Fluorescence mounting medium (DakoCytomation GmbH, Hamburg, Germany; cat. no. S3023).
7. 0.5% BSA in PBS for dilution of secondary antibodies.

Table 1
Primer Sequences, Annealing Temperature, and the Length of the Amplified Fragment Applicable for RT-PCR Amplification of Pancreas-Specific Genes

Gene	Primer sequence (forward/reverse)	Annealing temperature	Product size (bp)
Cytokeratin 19	5′-CTGCAGATGACTTCAGAACC	62°C	299
	5′-GGCCATGATCTCATACTGAC		
Glut-2	5′-TTCGGCTATGACATCGGTGTG	60°C	556
	5′-AGCTGAGGCCAGCAATCTGAC		
IAPP	5′-TGATATTGCTGCCTCGGACC	65°C	233
	5′-GGAGGACTGGACCAAGGTTG		
Insulin	5′-GTGGATGCGCTTCCTGCCCCTG	64°C	288
	5′-ATGCTGGTGCAGCACTGA		
Isl-1	5′-GTTTGTACGGGATCAAATGC	60°C	514
	5′-ATGCTGCGTTTCTTGTCCTT		
Nestin	5′-CTACCAGGAGCGCGTGGC	60°C	220
	5′-TCCACAGCCAGCTGGAACTT		
Ngn3	5′-TGGCGCCTCATCCCTTGGATG	60°C	157
	5′-AGTCACCCACTTCTGCTTCG		
Pax4	5′-ACCAGAGCTTGCACTGGACT	60°C	300
	5′-CCCATTTCAGCTTCTCTTGC		
Pax6	5′-TCACAGCGGAGTGAATCAG	58°C	332
	5′-CCCAAGCAAAGATGGAAG		
Pdx1	5′-CTTTCCCGTGGATGAAATCC	60°C	230
	5′-GTCAAGTTCAACATCACTGCC		
β-Tubulin	5′-TCACTGTGCCTGAACTTACC	60°C	317
	5′-GGAACATAGCCGTAAACTGC		

2.6. Enzyme-Linked Immunosorbent Assay

1. Solution A: dissolve 34.6 g sodium chloride, 1.8 g potassium chloride, 0.8 g potassium dihydrogen phosphate, 1.9 g calcium chloride, and 1.5 g magnesium sulfate in 1 L distilled water and mix.
2. Solution B: dissolve 13 g sodium hydrogen carbonate in 1 L distilled water and mix.
3. Krebs' ringer bicarbonate HEPES (KRBH) buffer: mix 200 mL solution A, 160 mL solution B, 2.4 g HEPES, and 2 g BSA; bring to a final volume of 1 L with distilled water, adjust to pH 7.4, and sterilize through a 0.22-µm filter. Prepare fresh every time.
4. 10 m*M* tolbutamide: dissolve 27 mg tolbutamide (Sigma, cat. no. T-0891) in 10 mL dimethyl sulfoxide (Sigma, cat. no. D2650).
5. KRBH buffer with 2.5 m*M* glucose: dissolve 45 mg glucose in 100 mL KRBH buffer and sterilize through a 0.22-µm filter.
6. KRBH buffer with 5.5 m*M* glucose: dissolve 100 mg glucose in 100 mL KRBH buffer and sterilize through a 0.22-µm filter.
7. KRBH buffer with 27.7 m*M* glucose: dissolve 500 mg glucose in 100 mL KRBH buffer and sterilize through a 0.22-µm filter.
8. KRBH buffer with 5.5 m*M* glucose and 10 µ*M* tolbutamide: dissolve 100 mg glucose and 100 µL 10 m*M* tolbutamide in 100 mL KRBH buffer and sterilize through a 0.22-µm filter.

9. Trypsin-EDTA (1:1) solution (*see* **Subheading 2.1.**, **item 6**).
10. Acid ethanol: 1 *M* hydrochloric acid:absolute ethanol (1:9).
11. Insulin ELISA (Mercodia, Uppsala, Sweden; cat. no. 10-1149-01).
12. Bradford assay (Bio-Rad Laboratories, München, Germany; cat. no. 500-0006).

2.7. Equipment

1. 35-, 60-, 100-mm tissue culture plates.
2. Bacteriological Petri dishes: 60 mm for embryoid body (EB) mass culture, 100 mm for EB hanging drop culture.
3. Pasteur pipets and 2-, 5-, 10- and 25-mL pipets.
4. Thoma chamber (Schütt Labortechnik, Göttingen, Germany).
5. Tissue culture incubator with 37°C and 5% CO_2 atmosphere.
6. Disposable cell scraper.
7. Smart Spec 3000 (Bio-Rad Laboratories).
8. PCR apparatus: mastercycler gradient (Eppendorf, Hamburg, Germany).
9. 0.6- and 1.5-mL microtubes and 20-, 100- and 1000-µL filter tips.
10. Silanized 1.5-mL microtubes (Heinemann, Duderstadt, Germany) and silanized 10-, 100-, and 1000-µL tips (Heinemann).
11. Electrophoresis equipment (Bio-Rad Laboratories).
12. Cover slips (16 × 16 mm and 12 mm ∅; Schütt Labortechnik) and slides.
13. ELISA reader (Bio-Rad Laboratories).
14. Inverted confocal laser scanning microscope LSM 510 META (Carl Zeiss, Jena, Germany).

3. Methods

3.1. ES Cell Culture (see Note 4)

1. Culture ES cells on a feeder layer in 60-mm tissue culture dishes in ES cell culture medium (*see* **Subheading 2.1., item 4**).
2. Change the medium 1–2 h before passage.
3. Aspirate the medium and quickly rinse the dish with 2 mL trypsin-EDTA (1:1) to remove remaining serum-containing medium.
4. Add 2 mL trypsin-EDTA (or the amount sufficient to cover the whole surface of the Petri dish) and incubate at room temperature for 30–60 s.
5. Remove carefully the trypsin-EDTA (1:1) solution and add 2 mL fresh ES cell culture medium.
6. Resuspend the cell population with a 2-mL glass pipet into a single-cell suspension and split 1:3 to freshly prepared feeder layer plates.

3.2. Generation of ES Cell-Derived Progenitor Cells (see Note 5)

1. Trypsinize ES cells as described in **Subheading 3.1.** and resuspend the single-cell solution in differentiation medium 1.
2. Prepare a cell suspension containing 600 ES cells in 20 µL of differentiation medium 1 (*see* **Subheading 2.2., item 3**).
3. Place single drops (n = 50–60) of 20 µL ES cell suspension onto the lids of 100-mm bacteriological Petri dishes containing 10 mL PBS and put the lids upside down on the plates.
4. Cultivate ES cells in hanging drops for 2 d. The cells aggregate and form one EB per drop.
5. Rinse the aggregates carefully from the lids with 2 mL differentiation medium (*see* **Note 9**, and **Subheading 2.3., items 14** and **15**) transfer the EBs into 60-mm bacteriological Petri dishes with 5 mL differentiation medium 1, and continue cultivation in suspension for 3 d.

6. Prepare gelatin-coated tissue culture plates by adding a sufficient amount of 0.1% gelatin to cover the whole surface of the dish; incubate for 1 h or overnight at 4°C. For immunofluorescence analysis, place cover slips on the bottom of the dish before gelatin coating. Aspirate gelatin solution before use.
7. Add a sufficient amount of differentiation medium 1 and transfer EBs onto the culture plates. Plate 20–30 EBs per 60-mm and 5–10 EBs per 35-mm tissue culture dishes (*see* **Note 6**).
8. Change the medium every second to third day until 9 d after EB plating (= 5 + 9d).

3.3. Induction of Pancreatic Differentiation (see Notes 7 and 8)

1. Prepare poly-L-ornithine/laminin-coated dishes:
   a. Add sterile poly-L-ornithine solution to the culture dishes and incubate for 3 h at 37°C. For immunofluorescence analysis, place cover slips on the bottom of the dish before adding of the poly-L-ornithine solution.
   b. Aspirate poly-L-ornithine solution, wash three times with distilled water and incubate with 5 mL distilled water at room temperature for 12 h.
   c. Rinse the dishes three times with distilled water and dry at 40°C.
   d. Incubate with laminin solution at 37°C for 3 h.
   e. Aspirate laminin solution and rinse the dishes twice with PBS.
2. Aspirate the medium from 60-mm tissue culture plates containing differentiating EB outgrowths; quickly rinse the cultures with 2 mL PBS.
3. Add a sufficient amount of trypsin-EDTA (1:1) to cover the whole surface and incubate at room temperature for 30–60 s.
4. Carefully remove the trypsin-EDTA solution and gently mechanically detach cells with the cell scraper.
5. Add 4 mL freshly prepared pancreatic differentiation medium (*see* **Note 9**) supplemented with 10% FCS, gently resuspend cells with a 5-mL glass pipet to obtain a suspension containing single cells and small clusters and plate 1 mL onto four freshly prepared poly-L-ornithine/laminin-coated 60-mm Petri dishes.
6. Add 3 mL pancreatic differentiation medium supplemented with 10% FCS and incubate overnight.
7. The next day, ensure that all cells attached, aspirate medium, and add pancreatic differentiation medium (without FCS).
8. Change the medium every second to third day. At 1 d before analysis, change medium.

3.4. RT-PCR Analysis (see Note 10)

3.4.1. Preparation of Cell Samples (see Note 11)

1. Discard the medium and wash twice with PBS.
2. Add 400 µL RNA lysis buffer per 60-mm culture dish. Allow the lysis buffer to spread across the surface of the dish and transfer the lysate into a 1.5-mL microtube (*see* **Note 12**).
3. Store samples at –20 or –80°C.

3.4.2. Isolation of Total RNA (see Note 13)

1. Thaw lysate (400 µL) and vortex for 15 s.
2. Add 40 µL (1/10 vol) of 2 *M* Na-acetate at pH 4.0. Mix carefully.
3. Add 400 µL acidic phenol and vortex vigorously.
4. Add 80 µL chloroform-isoamylalcohol (24:1) and vortex again.
5. Store for 15 min on ice.

6. Separate the organic and aqueous phases by centrifugation at 16,000*g* for 10 min at room temperature (*see* **Note 14**).
7. Transfer the upper aqueous phase carefully to a fresh tube, add an equal volume of isopropanol, and mix well. Store for 1 h at –20°C.
8. Centrifuge at 16,000*g* for 10 min at room temperature. Carefully discard the supernatant.
9. Dissolve the pellet in 300 µL lysis buffer. If the pellet is difficult to dissolve, then heat to 65°C for several min. Add an equal volume (300 µL) isopropanol and mix well. Store at –20°C for 1 h.
10. Centrifuge at 16,000*g* for 10 min at room temperature. Carefully discard the supernatant.
11. Wash the pellet with 500 µL 75% ice-cold ethanol, vortex briefly, recentrifuge at 16,000*g* for 10 min, discard supernatant, and allow the pellet of nucleic acid to dry in the air.
12. Dissolve RNA pellet in 10–30 µL DEPC-H_2O and freeze at –80°C.
13. Dilute 1 µL RNA with 100 µL DEPC-H_2O, measure OD_{260} and the concentration of RNA using a suitable spectrophotometer, adjust all samples to the same RNA concentration (i.e., 0.2–0.3 µg/µL) with DEPC-H_2O, and measure again to confirm the same RNA concentration of all samples.

3.4.3. RT Reactions (see ***Notes 15*** *and* ***16***)

1. Label one PCR reaction tube for each sample and appropriate controls. Add the same amount of RNA (0.5–1.0 µg in 3 µL) to each tube.
2. Prepare the following RT master mix for 25 reactions (or a smaller quantity as required): 261.25 µL DEPC-H_2O, 100 µL 5X reaction buffer, 20 µL 10 m*M* dNTPs mix, 12.5 µL RNase inhibitor, 25 µL Oligo $d(T)_{18}$, and 6.25 µL RevertAis M-MuLV RT to a total volume of 425 µL.
3. Add 17 µL RT master mix to each tube, mix carefully, and centrifuge briefly.
4. Transfer tubes to a thermal cycler, perform RT reactions for 1 h at 42°C, and then heat to 99°C for 5 min.
5. Cool the samples to 4°C.

3.4.4. Polymerase Chain Reactions (see ***Notes 16*** *and* ***17***)

1. Prepare a PCR master mix for 25 reactions (or a smaller quantity as required) containing 385 µL DEPC-H_2O, 62.5 µL 10X PCR buffer, 45 µL 25 m*M* $MgCl_2$, 40 µL 10 m*M* dNTPs mix, 25 µL 10 µ*M* 5′ sense primer of target gene, 25 µL 10 µ*M* 3′ antisense primer of target gene, and 6.25 µL *Taq* DNA polymerase to a total volume of 587.5 µL.
2. Label new PCR reaction tubes and add 1.5 µL RT reaction product to each tube as template DNA.
3. Add 23.5 µL PCR master mix to each tube, mix by vortexing, and centrifuge briefly.
4. Transfer tubes to thermal cycler. Amplify the complementary DNA through 25–40 thermal cycles. Standard conditions are denaturation at 95°C for 40 s, annealing at 55–68°C for 40 s, and extension at 72°C for 40 s (*see* **Note 18**).
5. Cool the samples to 4°C and store at –20°C.

3.4.5. Electrophoresis

1. Add 5 µL loading buffer to 25 µL PCR reaction.
2. Separate half of each PCR reaction (15 µL) and 10 µL of DNA ladder (indicator of product's size) by electrophoresis on a 2% agarose gel in 1X TBE supplemented with 0.35 µg/mL ethidium bromide at 5–10 V/cm for 70–100 min.
3. Illuminate the gel by ultraviolet light and obtain a digital image.

Table 2
Primary Antibodies and Fixation Methods Used for the Detection of Pancreatic Cells by Immunofluorescence

Protein	Antibody isotype	Working dilution	Supplier	Fixation	
				Met:Ac	PFA
Carbonic anhydrase II	Rabbit IgG	1:200	Abcam	–	+
C-peptide	Guinea pig IgG	1:100	Linco	–	+
Cytokeratin 18	Mouse IgG	1:100	Sigma	+	–
Cytokeratin 19	Mouse IgM	1:100	Chemicon	+[a]	+[b]
Glucagon	Rabbit IgG	1:100	Abcam	–	+
Insulin	Mouse IgG	1:40	Sigma	–	+
Isl-1	Rabbit IgG	1:200	Abcam	+	–
Nestin	Mouse IgG	1:3	Hybridoma Bank	+	+

Met:Ac, methanol:acetone solution; PFA, paraformaldehyde solution.
[a]Filament structures.
[b]Dot-like structures.

3.5. Immunofluorescence Analysis (see Note 19)

1. Rinse cover slips containing ES-derived cells twice with PBS.
2. Fix cells on cover slips with 4% PFA in PBS at room temperature for 15–20 min or alternatively with methanol:acetone (7:3) at –20°C for 10 min (depending on the antibody used; *see* **Table 2**).
3. Rinse cover slips twice with PBS at room temperature for 5 min.
4. Incubate the cells with 10% goat serum or 1% BSA in PBS in a humidified chamber at room temperature for 30 min to prevent unspecific immunostaining (*see* **Note 20**).
5. Incubate with the primary antibody at 37°C for 60 min or at 4°C overnight (dilutions of selected antibodies are indicated in **Table 2**).
6. Rinse cover slips with PBS three times at room temperature for 5 min.
7. Incubate with fluorescence-labeled specific secondary antibody (depending on the primary antibody) diluted in PBS with 0.5% BSA in a humidified chamber at 37°C for 45–60 min.
8. Incubate with 5 µg/mL Hoechst 33342 in PBS for 10 min at room temperature to label the cell nuclei.
9. Rinse cover slips twice with PBS at room temperature for 5 min.
10. Rinse cover slips quickly with distilled water at room temperature.
11. Embed cover slips in mounting medium and analyze immunolabeled cells with a conventional fluorescence or confocal laser scanning microscope (*see* **Note 21**).

3.6. Insulin ELISA (see Note 22)

1. Wash cells five times with PBS and preincubate in freshly prepared KRBH buffer with 2.5 m*M* glucose for 90 min at 37°C.
2. Replace KRBH buffer by either KRBH buffer containing 27.7 m*M* glucose or 5.5 m*M* glucose and 10 µ*M* tolbutamide (*see* **Note 22**).
3. Collect supernatant and store at –20°C for the determination of insulin release.
4. Dissociate cells by treatment with trypsin-EDTA (1:1) for 3 min and centrifuge.
5. Extract proteins with acid ethanol by overnight incubation at 4°C and after cell sonification and store at –20°C for determination of total cellular insulin and protein content.
6. Perform ELISA according to manufacturer's recommendations.

7. Determine total protein content with protein Bradford assay according to manufacturer's recommendations.

4. Notes

1. All reagents and materials are prepared sterile. Solutions are routinely sterilized through a 0.22-µm filter or autoclaved as indicated. Distilled water is prepared by double distillation and osmosis (Milli-Q water).
2. Ethidium bromide is carcinogenic. Use nitrile gloves and dispose all contaminated tips, agarose gels, and buffers separately.
3. PFA is toxic. Work under the hood and use gloves.
4. Reproducibility of ES cell differentiation is strongly dependent on the quality of ES cells used. To keep ES cells in the undifferentiated state, cells must be cultured at relatively high density and in the presence of MEF feeder cells or LIF. Good-quality FCS is critical for long-term culture of ES cells and for proper differentiation. Because ES cells divide every 12–15 h, the culture medium should be changed daily, and the cells have to be passaged every 24–48 h on freshly prepared and inactivated MEF feeder layers (for preparation, *see* **ref.** ***14***). For passaging, ES cells have to be dissociated by careful treatment with trypsin-EDTA solution. If one or more of these requirements is not met, then ES cells may differentiate and be unsuitable for differentiation studies.
5. For differentiation induction, remove feeder layer cells and LIF. The procedure for the generation of progenitor cells of all primary lineages (= multilineage progenitors) follows two steps: (1) ES cells are cultivated to form three-dimensional aggregates called EBs; and (2) EBs are plated onto adhesive substrates to allow expansion and spontaneous differentiation of cells. For differentiation of ES cells into early multilineage progenitors, ES cells are cultivated in EBs by the hanging drop method ***(14)*** or by mass culture in bacteriological plates ***(15)***. The advantage of the hanging drop method is a low variation of the size of EBs because of a controlled number of ES cells within the EBs and a better reproducibility of differentiation. EB formation for 5 d follows the development of cells of all three primary germ layers.
6. After suspension culture, EBs must be plated onto an adhesive surface at a proper density with homogeneous distribution. The distance between individual EBs should be sufficient to allow cells to proliferate and to migrate for several days after plating. If the EBs were plated at too high density, then differentiation may be inhibited. Continued culture of EB outgrowths results in multilayered cell clusters.
7. Spontaneous differentiation of ES cells results in the generation of cellular derivatives of all primary germ layers, but the proliferation and differentiation of specific progenitor cells is promoted by cultivation in the presence of specific growth and differentiation factors. The following parameters affect the efficiency of differentiation of ES-derived progenitors into pancreatic cells: (1) dissociation of the compact structure of the EB outgrowths (cell-to-cell interactions within cells of EB outgrowths may influence the fate of progenitor cells); (2) a suitable adhesive surface (extracellular matrix factors determine adhesion, proliferation, and migration of specific progenitor cells after replating of EB-dissociated cells); (3) cell density after replating (to support the proliferation and migration of a specific progenitor population, the number of cells should be optimal to prevent overgrowth resulting in metabolic starvation, necrosis, and cell death. If the initial amount of cells is low, then the threshold level of autocrine factors may be too low. As a consequence, the low growth factor activity and reduced cell-to-cell contacts may result in poor differentiation of specific cell types).
8. To increase the number of ES-derived pancreatic cells, the use of transgenic ES cells constitutively expressing pancreatic developmental control genes is recommended. We have shown

that constitutive expression of *Pax4* in (R1) ES cells significantly upregulates pancreatic β-cell-specific messenger RNA (mRNA) and protein levels, resulting in an increased number of insulin-expressing cells *(7)*.

9. The differentiation medium for the generation of pancreatic cells contains factors required for cell survival, such as progesterone, putrescine, insulin, sodium selenite, and transferrin, as well as factors promoting pancreatic differentiation, such as nicotinamide *(16)* and laminin *(17)*. The pancreatic differentiation medium is serum free. Coating of the tissue culture plates with poly-L-ornithine/laminin is necessary for pancreatic differentiation. The application of other substances promoting pancreatic differentiation, such as glucagonlike peptide 1 *(18)* and growth inhibitors *(19)* may further support pancreatic differentiation.
10. Use gloves and filter tips throughout the whole procedure.
11. mRNA isolation from small samples of cells can also be performed using the Dynabeads mRNA DIRECT microkit (Dynal Biotech, Oslo, Norway).
12. Do not leave RNA lysis buffer in culture dishes longer than 5 min because polystyrene is not resistant to lysis buffer.
13. The method described here is based on the use of a chaotropic agent (guanidine salt) for disruption of cells and inactivation of RNases *(20)*.
14. Never mix and disturb the organic and the aqueous phases.
15. For RT-PCR, rTth DNA polymerase can also be used as both RT and DNA polymerase *(21)*. In this case, the components of both RT and PCR master mix are different from using MuLV RT and *Taq* DNA polymerase.
16. All RT and PCR solutions are available from commercial suppliers in ready-to-use form. RT reactions are performed in 20 µL of reaction volumes using 0.5-mL microcentrifuge tubes.
17. PCRs are performed in 25 µL of reaction volumes using 0.5-mL microcentrifuge tubes.
18. The conditions depend on the primers and thermal cycler used.
19. The presence of insulin in the culture medium may lead to an overestimation of its endogenous production. It has been shown that ES-derived pancreatic cells were immunopositive for insulin as a consequence of insulin uptake from the medium *(22)*. For pancreatic differentiation, therefore, C-peptide, a by-product of insulin synthesis, serves as a reliable marker of insulin production. Demonstration of pancreatic differentiation by immunofluorescence requires parallel RT-PCR analysis, including a comparison of various markers.
20. Unspecific binding of primary and especially secondary antibodies to the cells should be blocked by incubation with serum proteins of animal species not used for the generation of the primary antibody. Specificity of immunostaining could be demonstrated by the absence of signals after incubation with PBS or with control antibodies instead of the specific primary antibody.
21. For immunofluorescence analysis of ES-derived isletlike clusters, it is necessary to use a confocal laser scanning microscope (CLSM) because the clusters represent three-dimensional aggregates, which require an extended depth of focus. Therefore, isletlike clusters should be scanned in thin sections (0.5–10 µm) using the appropriate filter combinations depending on the fluorescent dyes applied; by using appropriate computer software, the sections should be processed to generate three-dimensional projection.
22. The analysis of differentiated pancreatic endocrine cells should include the determination of insulin production as a functional assay. The intracellular insulin content can be measured by commercialized specific insulin ELISA. In addition, the glucose responsiveness should be tested. For this purpose, insulin release in the presence of low (5.5 m*M*, as a control) and high (27.7 m*M*) glucose concentration is determined. Tolbutamide (10 µ*M*), a sulfonylurea known to stimulate insulin secretion, together with 5.5 m*M* glucose can also be

used. However, failure of glucose response may be dependent on insufficient maturation during differentiation. Such effects were already described during pancreatic differentiation of mouse ES cells, in which insulin was secreted in response to glucose at an advanced stage of 32 d of differentiation but not at d 28 *(7)*.

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26

Derivation and Characterization of Hepatocytes From Embryonic Stem Cells In Vitro

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Summary

From a therapeutic perspective on liver, the use of embryonic stem (ES) technology in the generation of a large number of high-functional hepatocytes developed from ES cells for cell transplantation is anticipated. We have explored a three-dimensional culture system in which hepatocytes differentiated from mouse ES cells by transfection with the hepatocyte nuclear factor-3β possess high metabolic functions that can be maintained long term.

Key Words: Albumin; chitin; complement C3; differentiation; ES cells; glycogen; hepatocytes; HNF-3β; P450; PEPCK; PXMP1-L; three-dimensional culture; transfection; triacyglycerol; urea.

1. Introduction

In most mammals, hepatocytes initially develop from the embryonic endoderm by a variety of inductive signaling types from the precardiac mesoderm, septum transversum mesenchyme, and endothelial cells *(1,2)*. Transfection of embryonic stem (ES) cells with hepatocyte nuclear factors (HNF)-3β has been shown to induce expression of endoderm marker genes as well as HNF-1 and HNF-4 *(3,4)*. Hepatocyte differentiation is coordinately regulated by multiple hepatocyte transcription factors such as HNF-1α, -1β, -3α, -3β, -γ, -4α and -6 as well as Gata 4 *(5–7)*. HNF-α and HNF-β are expressed throughout the primitive endoderm *(8,9)*, HNF-4α plays an important role in hepatocyte differentiation *(10)* and regulation of many hepatocyte genes *(11)*. Fibroblast growth factors (FGF) 1 and 2 from the precardiac mesoderm have been known to induce hepatic development from the endoderm *(1)*.

To promote hepatic development from the endoderm, HNF-3β-transfected ES (HTC) cells are cultured in hepatocyte culture medium with FGF-2. Supplementing of nicotinamide, dexamethasone, and L-ascorbic-2-phosphate in hepatocyte culture medium may be effective at enhancing the survival of hepatocytes *(12)*, stimulating the growth

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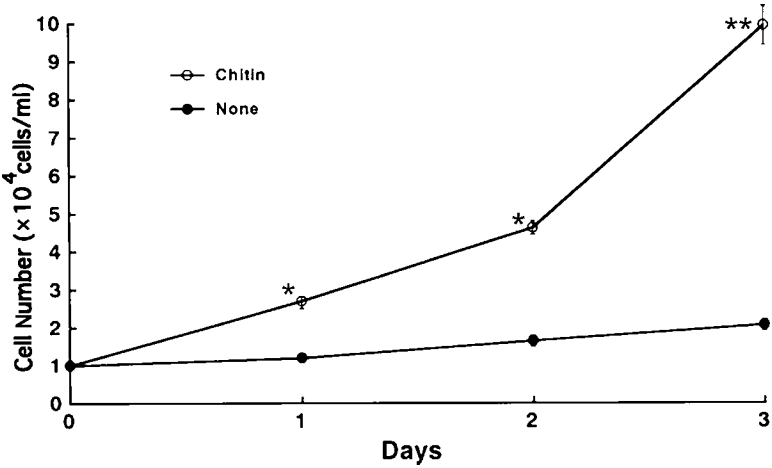

Fig. 1. Proliferation of hepatocyte nuclear-3β-transfected embryonic stem (HTC) cells. The cell number of HTC spheroids in the flask (closed circle) and flasks containing chitin fibers (open circle) were counted for 3 d. (Reproduced with permission from **ref.** *19*.)

of regenerating hepatocytes *(13)*, and amplifying a cytoprotective effect on hepatocytes *(14,15)*, respectively.

It has been generally difficult for primary hepatocytes to maintain continuous cell proliferation and metabolic functions for a long-term culture; therefore, a three-dimensional culture system using matrices such as poly-(2-hydroxyl methacrylate) *(15)*, Matrigel *(16)*, hydrophilic polyester fabric *(17)*, and alginate sponges *(18)* is necessary for long-term maintenance of metabolic functions and growth of hepatocytes. In our study, HTC cells in a three-dimensional culture system using chitin fibers could maintain liver-specific metabolic functions long term *(19)*. Chitin fibers, which are extracted from crabs, lead to an increase in the number of HTC spheroids (**Fig. 1**). In this chapter, we describe our protocol for in vitro differentiation of ES cells into hepatocytes with liver-specific metabolic functions and hepatocytelike morphometric characterizations, as well as for the structural and functional characterization of the ES-derived hepatocytes.

2. Materials

2.1. ES Cell Culture

1. ES cell medium: to 500 mL Dulbecco's modified Eagle's medium with 4.5 g/L D-glucose (Gibco BRL, Grand Island, NY; cat. no. 11960-044), add 5 mL 10 m*M* minimum essential medium (MEM) nonessential amino acids (Gibco BRL, cat. no. 11140-50), 5 mL 200 m*M* L-glutamine (Gibco BRL, cat. no. 25030-081), 5 mL 100 m*M* MEM sodium pyruvate (Gibco BRL, cat. no. 11360-50), 500 µL 0.1 *M* 2-mercaptoethanol (Sigma, St. Louis, MO; cat. no. M-7522), 50 µL 10^7 U/mL leukemia inhibitory factor (Gibco BRL, cat. no. ESG1107), and 75 mL fetal bovine serum (Gibco BRL, cat. no. 16141-079).
2. Calcium- and magnesium-free Dulbecco's phosphate-buffered saline (PBS) (Sigma, cat. no. D7030).
3. 0.1% gelatin in sterile water (Sigma, cat. no. G1890).

4. Trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Gibco BRL, cat. no. 25200-056).
5. Six-well multidishes (Nunc, Rochester, NY; cat. no. 152795).

2.2. Transfection and Construction of the Plasmid HNF-3β

1. pGEM^2-2-HNF-3β plasmid DNA (Dr. R. H. Costa, University of Illinois).
2. EcoR I (Takara, Otsu, Japan; cat. no. 1040A).
3. 10X H buffer (Takara; buffer supplied with restriction enzyme EcoR I).
4. Agarose (Nacalai tesque, Kyoto, Japan; cat. no. 011-53).
5. 10X loading buffer (Takara; buffer supplied with restriction enzyme EcoR I).
6. Submarine-type electrophoresis system (Advance, Tokyo, Japan; model no. Mupid-21).
7. 100-bp DNA ladder marker (Takara, cat. no. 3407A).
8. 10 mg/mL ethidium bromide (Bio-Rad, Hercules, CA; cat. no. 161-0433).
9. Ultraviolet light source (ATTO, Tokyo, Japan; model no. DT20MP).
10. DNA purification kit (Bio-Rad, cat. no. 732-6010).
11. pPyCAG-hygro (Dr. H. Niwa, Center for Developmental Biology, Japan).
12. BstX I (Takara, cat. no. 1027A).
13. Calf intestinal alkaline phosphatase kit (Takara, cat. no. 2250A).
14. Phenol/chloroform/isoamyl alcohol (25:24:1) (Nippon gene, Tokyo, Japan; cat. no. 311-90151).
15. T4 DNA ligase (Takara, cat. no. 2011A).
16. 10X ligation buffer (Takara; buffer supplied with T4 DNA ligase).
17. TE buffer: for 20 mL, dissolve 100 µL 2 *M* Tris-HCl (pH 8.0) and 40 µL 0.5 *M* EDTA (pH 8.0) into 19.9 mL sterile water.
18. Hygromycin B (Invitrogen, Carlsbad, CA; cat. no. 10687-010).
19. Luria-Bertani (LB) medium: 10 g NaCl, 10 g bacto tryptone, and 5 g yeast extract in 1 L distilled water (dH_2O).
20. Bacto-agar (BD Difco, Livonia, MI; cat. no. 0140-01).
21. *Escherichia coli* DH5α-competent cells (Invitrogen, cat. no. 10687-010).
22. Alkaline lysis solution 1: for 100 mL, dissolve 2.5 mL 1 *M* Tris-HCl (pH 8.0), 2 mL 0.5 *M* EDTA, and 0.9 g glucose into 95 mL sterile water.
23. Alkaline lysis solution 2: for 100 mL, add 0.8 g NaOH and 1 g sodium dodecyl sulfate into 100 mL sterile water.
24. Alkaline lysis solution 3: for 100 mL, mix 60 mL 5 *M* potassium acetate, 11.5 mL glacial acid, and 28.5 mL dH_2O.
25. Wizard purefection plasmid DNA purification kit (Promega, Madison, WI; cat. no. A2150).
26. Gene Pulser transfection apparatus (Bio-Rad, cat. no. 165-2075).
27. Hanks balanced salt solution (HBSS) (Invitrogen, cat. no. 14170-120).

2.3. Differentiation Into Hepatocytes

1. Differentiation medium: to 438 mL 500 mL α-MEM medium (Gibco BRL, cat. no. 12571-063), add 50 mL fetal bovine serum (Sigma, cat. no. F9423), 25 µg FGF-2 (Roche Applied Science, Mannheim, Germany; cat. no. 1123149), 0.5 mL 10^{-4} *M* dexamethasone (Sigma, cat. no. D2915), 1 mL 100 m*M* L-ascorbic-2-phosphate (Sigma, cat. no. A8960), 5 mL 1 *M* nicotinamide (Sigma, cat. no. N0636), and 5 mL penicillin-streptomycin (10,000 U/mL-10,000 µg/mL; Gibco BRL, cat. no. 15140-122).
2. 96-well round-bottom Sumilon cell-tight spheroid plates (Sumitomo Bakelite, Tokyo, Japan; cat. no. MS-0096s) and 60-mL plastic flasks (Techno Plastic Products AG, Trasadingen, Switzerland; cat. no. 90026).
3. Defatted chitin fibers were obtained from T. Asami (Omikenshi, Osaka, Japan). Sterilize by autoclaving (*see* **Note 1**).

2.4. Histology and Immunochemistry

1. 10% phosphate-buffered formalin at pH 7.4 (Wako Pure Chemicals, Osaka, Japan; cat. no. 068-03841).
2. 1 mg/mL α-amylase (1500–3000 U/mg; Sigma, cat. no. A6380) in 0.1 *M* phosphate buffer at pH 6.2.
3. 0.5% periodic acid solution (Wako Pure Chemicals, cat. no. 164-19705).
4. Shiff's reagent (Wako Pure Chemicals, cat. no. 191-08441).
5. Sulfurous acid solution (Wako Pure Chemicals, cat. no. 195-11955).
6. Mayer's hematoxylin solution (Wako Pure Chemicals, cat. no. 131-09655).
7. Xylene (Wako Pure Chemicals, cat. no. 245-00717).
8. Ethanol (EtOH) (Wako Pure Chemicals, cat. no. 050-00446).
9. Histomouse-plus kits (blocking solutions A and B, biotinylated second antibodies, substrate-chromogen mixture) (Zymed Laboratories, South San Francisco, CA; cat. no. 85-9541).
10. Horseradish peroxidase (HRP)-conjugated antimouse albumin antibodies (Bethyl Laboratories, Montgomery, TX; cat. no. A90-134P).
11. Rabbit anti-CK19 antibodies (gift from Dr. Lucie Germain).
12. Anti-mouse CK18 antibodies (Chemicon International, Temecula, CA; cat. no. MAB3234).

2.5. Reverse Transcriptase Polymerase Chain Reaction

1. Concanavalin A (Calbiochem, San Diego, CA; cat. no. 234567).
2. Phenobarbital (Sigma, cat. no. P5178).
3. Glucagon (Sigma, cat. no. G3157).
4. Fenofibrate (Sigma, cat. no. F6020).
5. Trizol reagent (Gibco BRL, cat. no. 15596-026).
6. SuperScript preamplification system (Gibco BRL, cat. no. 18089-011).
7. PCR medium: for 100 µL, mix 10 µL 100 m*M* Tris-HCl (pH 8.3), 10 µL 500 m*M* KCl, 10 µL 15 m*M* $MgCl_2$, 8 µL 2.5 m*M* of each dNTP, 0.5 µL 5 U/µL Takara *Taq* DNA polymerase (Takara, cat. no. R001A), and 61.5 µL sterile water.
8. PCR primer sets: Albumin *(4)*: 5′-TGAACTGGCTGACTGCTGTG-3′ and 5′-CATCCTTGGCCTCAGCATAG-3′, C3 *(20)*: 5′-CACCGCCAAGAATGCCTAC-3′ and 5′-GATCAGGTGTTTCAGCCG-3′, P450 Cyp3a11 *(21)*; 5′-TGAGGCAGAAGGCAAAGAAA-3′ and 5′-GGTATTCCATCTCCATCACA-3′, PEPCK; 5′-AGGCAGTGAGGAAGTTCGT-3′ (214-232 nucleotides) and 5′-TCTTCCCACAGGCACTAGG-3′ (993-1011 nucleotides), HNF-3β; 5′-AGAAGCAACTGGCACTGAAGGA-3′ (173-194 nucleotides) and 5′-GTAGTGCATGACCTGTTCGTAG-3′ (615-636 nucleotides), PXMP1-L *(22)*; 5′-CTTCAGACCCAGAGAGAGCTG-3′ and 5′-CCCGTGTTGCCTGTGATGAGC-3′, and β-actin *(4)*; 5′-TGAAACAACATACAATTCCATCATGAAGTGTGAC-3′ and 5′-AGGAGCGATAATCTTGATCTTCATGGTGCT-3′.
9. Thermal cycler (MJ Research, South San Francisco, CA; model no. PTC-200).
10. Agarose (Nacalai tesque, cat. no. 011-53).
11. Tris-borate-EDTA electrophoresis buffer: for 1000 mL, combine 100 mL 890 m*M* Tris-base, 100 mL 890 m*M* boric acid (pH 8.0), 4 mL 500 m*M* EDTA (pH 8.0), and 796 mL dH_2O.
12. DNA molecular weight markers (Takara, cat. no. 3407A).
13. Ethidium bromide (Bio-Rad, cat. no. 161-0433).
14. Horizontal gel electrophoresis apparatus (DNA Technologies, Gaithersburg, MD; model no. Mupid-21).

2.6. Urea Synthesis in HTC Cells

1. Ammonium chloride (Wako Pure Chemicals, cat. no. 017-02995).
2. DeterminerL UN kit (Kyowa Medix, Tokyo, Japan; cat. no. 32519-2).
3. Clinical analyzer (Hitachi, Hitachinaka, Japan; model no. 7170).

2.7. Triacyglycerol Synthesis in HTC Cells

1. Oleic acid solution: for 1 *M*, add 2.825 g oleic acid (Sigma, cat. no. O1383) to 10 mL PBS (Sigma, cat. no. D7030) containing 20 mg bovine serum albumin (Sigma, cat. no. A8806).
2. Insulin (Eli Lilly, Indianapolis, IN; cat. no. HI340).
3. Triacyglycerol E-test kit (Wako Pure Chemicals, cat. no. 433-40201).
4. Microplate reader (Corona Electric, Hitachinaka, Japan; model no. MTP-32).

2.8. Electron Microscopy

1. 0.2 *M* cacodylate buffer (pH 7.4): for 1000 mL, add 42.8 g cacodylic acid sodium salt (Nacalai Tesque, cat. no. 065-16) to 950 mL dH_2O, adjust to pH 7.4 with 37% hydrochloric acid (Wako Pure Chemicals, cat. no. 083-01066), and then make 1000 mL solution by adding distilled water suitably.
2. 1.5% glutaraldehyde (GA) solution: for 160 mL, add 10 mL 25% GA (Merck, Darmstadt, Germany; cat. no. 820603), 50 mL 0.2 *M* cacodylate buffer (pH 7.4), and 1.6 g sucrose (Wako Pure Chemicals, cat. no. 196-00015) to 100 mL dH_2O and store at 4°C.
3. 2% osmium tetroxide (OsO_4) solution: for 50 mL, add 1 g OsO_4 (Merck, cat. no. 124505) to 50 mL dH_2O.
4. 1% OsO_4 solution: for 2 mL, combine 1 mL 2% OsO_4 solution and 1 mL 0.2 *M* phosphate buffer (PB) at pH 7.4.
5. 0.2 *M* PB (pH 7.4): for 1000 mL, add 58.02 g disodium hydrogen phosphate 12-water acid (Wako Pure Chemicals, cat. no. 196-02835) and 5.92 g sodium dihydrogen phosphate dehydrate (Wako Pure Chemicals, cat. no. 192-02815) to 950 mL dH_2O and then make 1000 mL solution by adding distilled water suitably.
6. 0.01 *M* PBS: for 1000 mL, add 2.87 g disodium hydrogen phosphate 12-water, 0.33 g sodium dihydrogen phosphate dehydrate, and 8.5 g sodium chloride (Wako Pure Chemicals, cat. no. 191-01665).
7. 2% agar solution: for 100 mL, add 2 g agar (Wako Pure Chemicals, cat. no. 016-15812) to 100 mL 0.1 *M* PB at pH 7.4 (*see* **Note 2**).
8. 70, 90, 95, 99, and 100% EtOH (Wako Pure Chemicals, cat. no. 057-00456).
9. Epoxy resin: for 100 mL, add 46.6 mL Poly/Bed 812 resin (Polysciences, Warrington, PA; cat. no. 08791), 25 mL dodecenylsuccinic anhydride (Polysciences, cat. no. 00563), 28.4 mL nadic methyl anhydride (Polysciences, cat. no. 00886), and 1.7 mL 2,4,6-tri(dimethylaminomethyl) phenol (Polysciences, cat. no. 00553).
10. Mix solution of epoxy resin with acetone (1:1): for 10 mL, mix 5 mL epoxy resin and 5 mL acetone (Wako Pure Chemicals, cat. no. 016-00346).
11. Uranyl acetate (TAAB Laboratories Equipment, Berkshire, UK; cat. no. V001).
12. Lead citrate (TAAB Laboratories Equipment, cat. no. L018).

3. Methods

3.1. Gelatin Coating of Dishes

1. Add 3 mL 0.1% gelatin to each well of six-well multidish.
2. Aspirate after 5 min.

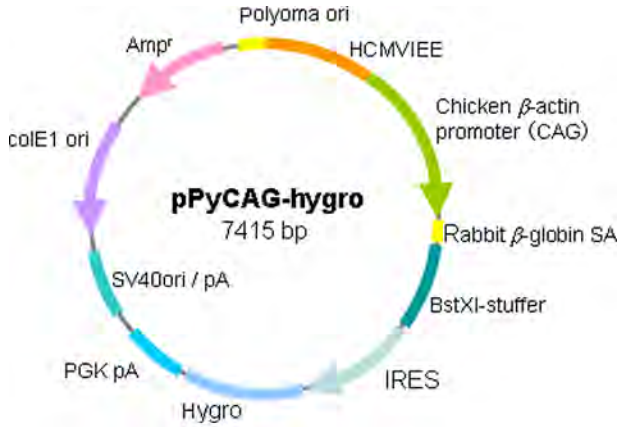

Fig. 2. Schematic representation of the expression vector pPyCAG-hygro. (Reproduced with permission from **ref.** *19*.) (Please *see* the companion CD for the color version of this figure.)

3.2. Maintenance of ES Cells

1. ES (CGR8) cells are cultured in the ES medium on gelatin-coated dishes without feeders (*see* **Note 3**).
2. Change ES cell medium daily until subconfluent.
3. Aspirate the medium, wash twice with PBS, and trypsinize with trypsin-EDTA solution at 37°C for 3–5 min.
4. Add cell medium and dissociate the ES cell clump by pipetting. Centrifuge at 500*g* for 5 min and resuspend the pellet in culture medium.
5. Seed ES cells (1×10^5 cells/well) in a new six-well multidish.
6. Cells should be passaged every 3 d.

3.3. Transfection and Construction of the Plasmid HNF-3β

3.3.1. Construction of HNF-3β Expression Vector Plasmid (*Fig. 2*)

1. Digest 10 µg pGM-2-HNF-3β plasmid DNA by EcoR I at 37°C for 2 h.
2. After digestion, electrophorese the plasmid DNA solution on a 2% agarose gel and extract HNF-3β complementary DNA fragment from the gel containing the expected band with a gel extraction kit; dissolve complementary DNA in TE buffer.
3. Digest 10 µg pPyCAG-hygro plasmid vector by BstX I at 45°C for 2 h.
4. After digestion, dephosphorylate the 5′ end of the vector plasmid by calf intestinal alkaline phosphatase, extract by phenol/chloroform/isoamyl alcohol (25:24:1), precipitate by 70% alcohol, and dry. Resuspend 100 ng of the pellet in TE buffer.
5. Add vector DNA, a fivefold molar-annealed oligonucleotide insert and 1 µL T4 DNA ligase to each tube. Ligate at 16°C for 15 h.
6. Mix 5 µL reaction solution and 100 µL *E. coli* DH5α-competent cells gently; keep on ice for 30 min and incubate at 37°C for 1 h.
7. Spread the cell suspension on an LB plate containing hygromycin B (50 µg/mL) and incubate at 37°C for 15 h.
8. Pick 20 single colonies and incubate in LB medium containing hygromycin B at 37°C for 15 h.

9. Collect *E. coli* in culture medium by centrifugation at 6000*g* for 10 min and purify the plasmid DNA by an alkaline prep method.
10. Check the insert in the vector by digestion of BstX I; carry out the large-scale culture and construct for the transfection-grade plasmid DNA using the Wizard purefection plasmid DNA purification system according to the manufacturer's instructions.

3.3.2. Transfection of ES Cells

1. Culture ES cells until the cells become confluent in a 10-cm dish and change the medium several hours before electroporation.
2. Harvest the ES cells with 0.25% trypsin, mix the cell suspension well in cold HBSS, and count the cell number with a hemocytometer. Prepare 0.4 mL cell suspension (1×10^7 cells).
3. Prepare 100 µL 25 n*M* HNF-3β plasmid vector dissolved in HBSS and mix well with the cell suspension prepared in **step 2** by repeated pipetting.
4. Transfer to a 0.4-cm Gene Pulser cuvet and leave on ice for 10 min. Electropolate with a Gene Pulser apparatus at 250 V and 960 µF. After electroporation, leave the cells on ice for 10 min.
5. Add 24 mL prewarmed ES cell medium, disperse the suspension into six gelatin-coated 6-cm Petri dishes, and incubate at 37°C in an incubator (5% CO_2).

3.3.3. Isolation of HNF-3β-Expressed ES Clones

1. At 24 h after electroporation, change the medium to ES cell medium supplemented with 200 µg/mL hygromycin B.
2. Change the medium every day to remove dead and unattached cells. After 6–10 d of the selection, ES cell colonies are 1–2 mm in diameter.
3. Under a dissecting microscope, use a 20-µL pipet tip to scrape a circle around the colony to be transferred. Set the pipettor to 10 µL and pipet the colony into the tip.
4. Transfer the colony into 50 µL 0.25% trypsin in a 96-well tissue culture plate. Digest for 5 min at room temperature. Pipet up and down to disperse the cells, transfer to 1 well of a 24-well tissue culture plate with 1 mL ES cell medium containing both leukemia inhibitory factor and hygromycin B, and incubate at 37°C (5% CO_2).
5. When visible growth of clones is indicated by a pH change in the medium, wash with PBS(–) and harvest by treating with 100 µL 0.25% trypsin.
6. Add the same volume of ES cell medium, pipet with pipettor, and then add 200 µL ES cell medium containing 20% dimethyl sulfoxide. Mix by pipetting and transfer the cell suspension into a cryotube.
7. Freeze the clones to ensure long-term availability. Leave the cryotube at 4°C for 60 min, followed by 2 h at –20°C and overnight at –80°C. Transfer the cryotube to –150°C deep freezer.

3.4. Differentiation of HTC Cells Into Hepatocytes (Fig. 3)

1. Culture HTC cells (2×10^4 cells/0.2 mL) in differentiation medium in 96-well, round-bottom plates for spheroid formation for 1 mo and change the medium every 2 d (*see* **Note 4**).
2. Transfer HTC spheroids into plastic flasks containing chitin fibers (50 mg/15 mL medium) for cell growth in the three-dimensional culture system and incubate in 15 mL differentiation cell medium without FGF-2 under an atmosphere of 5% CO_2 in air at 37°C for 4 mo (*see* **Note 5**).
3. Change the medium and passage spheroids into new flasks every 2 d.

3.5. Characterization of ES Cell-Derived Hepatocytes

3.5.1. Staining of Glycogen (see **Note 6**)

1. Fix with 10% phosphate-buffered formalin for 30 min.
2. Treated with α-amylase at 37°C for 30 min. Wash three times with water.

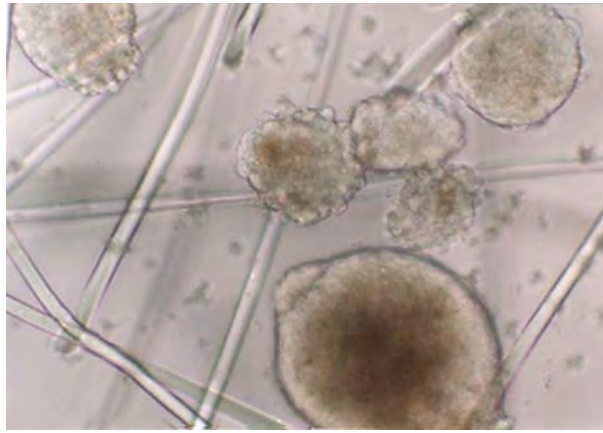

Fig. 3. Hepatocyte nuclear-3β-transfected embryonic stem cells were cultured in flasks containing chitin fibers at 3 m after the initial culture. Reproduced with permission from **ref. *19***.

3. Place slides in 0.5% periodic acid solution (PAS) for 10 min. Wash three times with water.
4. Stain in Shiff's reagent for 10 min.
5. Wash in two changes of sulfurous acid solution for 2 min each to remove any nonspecific stain.
6. Counterstain with Mayer's hematoxylin solution for 30 s. Wash with running tap water for 3 min.
7. Dehydrate through 95% alcohol and 100% alcohol to xylene.

3.5.2. Immunochemistry (see ***Note 7***)

1. Deparaffinize paraffin-embedded slides in xylene twice for 3 min.
2. Rinse in two changes of 100% alcohol for 3 min.
3. Rinse in two changes of 95% alcohol for 3 min.
4. Place slides in PBS for 5 min.
5. Add two drops blocking solution A and incubate for 10 min.
6. Wash with distilled water.
7. Add two drops blocking solution B and incubate for 10 min.
8. Wash with distilled water for 2 min and three times with PBS.
9. Apply two drops HRP-conjugated antimouse albumin antibodies (1:100 dilution), anti-CK18 antibodies (1:100 dilution), or anti-CK19 antibodies (1:100 dilution) and incubate at room temperature for 1 h.
10. Wash three times with PBS.
11. Apply two drops biotinylated secondary antibodies against the primary antibodies other than HRP-conjugated antimouse albumin antibodies and incubate at room temperature for 10 min.
12. Wash with distilled water.
13. Apply two drops enzyme conjugate and incubate at room temperature for 10 min.
14. Wash three times with PBS.
15. Apply two drops substrate-chromogen mixture and incubate for 10 min.
16. Wash with distilled water.

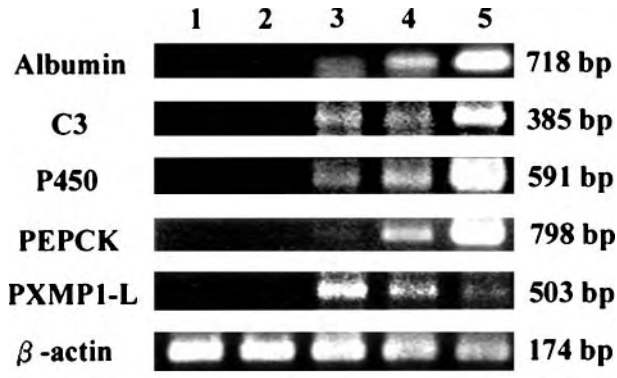

Fig. 4. Expression of differentiated hepatocyte-enriched protein markers in hepatocyte nuclear-3β-transfected embryonic stem (ES) cells (HTC) 1, ES cells; 2, HTC cells cultured in ES cell maintenance medium; 3, HTC cells (1 mo); 4, HTC cells (4 mo); 5, normal mouse liver. (Reproduced with permission from **ref.** *19*.)

3.6. Gene Expression of HTC Cells

3.6.1. Induction of mRNA

1. Induce complement C3 mRNA *(23)* by adding conditioned medium (50 µL/mL) from Concanavalin A (1 µg/mL)-stimulated mouse spleen cells to HTC cells cultured in differentiation medium.
2. Induce P450 mRNA *(24)* by culturing HTC cells with the addition of 100 µ*M* phenobarbital for 48 h.
3. Induce PEPCK mRNA *(25)* by incubating HTC cells with the addition of 20 n*M* glucagon for 2 h.
4. Induce PXMP1-L mRNA *(26)* by treating HTC cells with the addition of 250 µ*M* fenofibrate for 24 h.

3.6.2. RT-PCR Analysis (Fig. 4)

1. RT-PCR analyses are performed under the following conditions: 1 cycle at 94°C for 60 s followed by 30 cycles at 94°C for 60 s, annealing at 60°C for 60 s, at 54°C for HNF-3β, 72°C for 60 s, and a final extension for 15 min at 72°C.
2. The PCR products (10 µL) are electrophoresed in 2% agarose gel in Tris-borate-EDTA buffer and visualized by ethidium bromide staining.

3.7. Urea Synthesis in HTC Cells (Fig. 5A)

1. HTC cells (10^5 cells/well) are cultured in serum-free α-MEM medium in the absence or presence of 5, 10, and 20 m*M* ammonium chloride.
2. Mix culture medium (40 µL) with 2.8 mL reagent I from the Determiner LUN kit and incubate at 37°C for 5 min to remove endogenous ammonium by treatment with glutamate dehydrogenase.
3. Add 0.7 mL reagent II from the Determiner LUN kit to the mixture and incubate at 37°C for 5 min after shaking rigorously.
4. Determine the urea nitrogen level in the culture medium by monitoring absorbance at 340 nm.

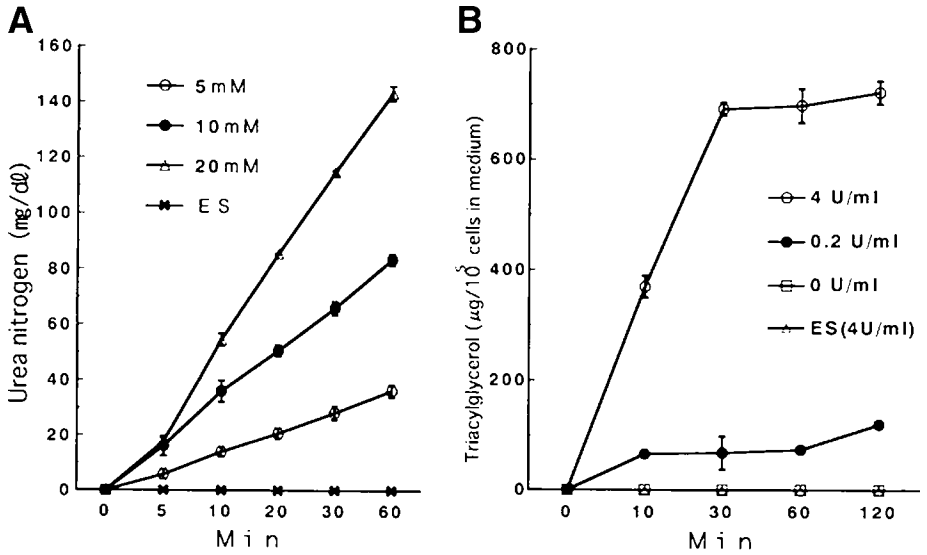

Fig. 5. The short time-course of urea and triacyglycerol synthesis in hepatocyte nuclear-3β-transfected embryonic stem (ES) cells (HTC). (**A**) HTC cells and ES cells (**x**) were cultured in serum-free minimum essential medium α-(MEM) medium in the absence or the presence of 5 (open circle), 10 (closed circle), and 20 (open triangle) m*M* ammonium chloride. (**B**) HTC cells and ES cells (open triangle) were incubated in serum-free α-MEM medium without (open square) or with 0.2 (closed circle) or 4 (open circle) U/mL insulin. (Reproduced with permission from **ref.** *19*.)

3.8. Triacyglycerol Synthesis in HTC Cells (Fig. 6)

1. Incubate HTC cells (10^5 cells/well) in serum-free α-MEM medium supplemented by 0.5 m*M* oleic acid in the absence or the presence of 0.2 or 4 U/mL insulin.
2. Collect 10 µL incubated medium according to the manufacturer's instructions.
3. Mix sample medium (10 µL) or control solutions (10 µL containing up to 596.1 µg glycerol) and the color former (200 µL) of triacylglycerol E-test kit.
4. Incubate the mixture at 37°C for 5 min.
5. Determine the absorbance at 600 nm.

3.9. Electron Microscopy

1. Fix the cells with 1.5% GA solution for 2 h at 4°C.
2. Centrifuge the cells at 200*g* for 10 min at 4°C.
3. Discard supernatant and postfix in 1% OsO_4 solution overnight at 4°C.
4. Centrifuge the cells at 200*g* for 10 min at 4°C.
5. Discard supernatant, suspend the pellet in 0.01 *M* PBS, and centrifuge the cell suspension at 200*g* for 10 min at 4°C. Repeat this process twice.
6. Discard supernatant and add 2% agar to the pellet. Keep the tube on ice for 1 h.
7. Cut the agar with cells into small pieces.
8. Dehydrate by passing through an EtOH series of 50, 70, 90, 95, and 99% EtOH for 2 min each and 100% EtOH three times for 5 min.

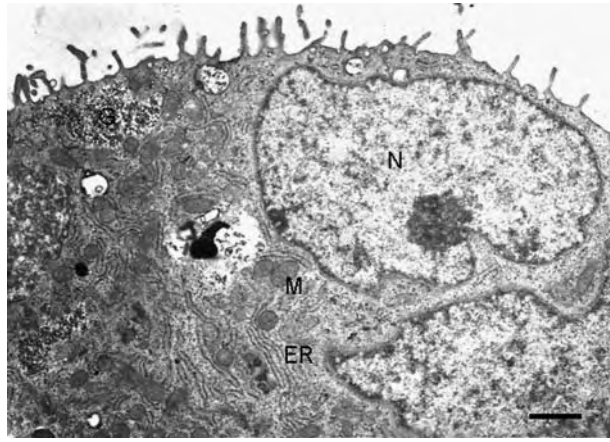

Fig. 6. Electron micrograph of hepatocyte nuclear-3β-transfected embryonic stem cells containing mitochondria (M), rough endoplasmic reticulum (ER), glycogen (G), and nucleus (N). Scale bar = 1 μm. (Reproduced with permission from **ref.** ***19***.)

9. Add mixed solution of epoxy resin with acetone to the cells and shake for 1 h at room temperature to enhance the penetration of epoxy resin into the samples.
10. Remove mixed solution, add epoxy resin, and shake overnight at room temperature, leading to an increase in the penetration of resin into the samples.
11. Remove epoxy resin and embed in fresh epoxy resin by keeping the cells at 60°C for 5 d.
12. Cut 70-nm thick ultrathin sections with an ultramicrotome; stain with saturated uranyl acetate for 30 min and saturated lead citrate for 5 min (*see* **Note 8**).
13. Observe with an electron microscope at 100 kV.

4. Notes

1. HTC cells do not attach to chitin fibers, which are extracted from crabs. The amount of chitin fibers reduces gradually by about 50% in the degradation process under cell culture for 1 mo.
2. We make this solution using a microwave oven and then maintain a temperature of 60°C to avoid solidification.
3. ES (CGR8) cells, established by Dr. H. Niwa, are able to keep growth in an undifferentiated state without feeder cells.
4. A single gross spheroid with an initial diameter from about 150 to 300 μm is formed in each well of 96-well, round-bottom Sumilon-tight spheroid plate for 3–5 d.
5. The cells in the outer layer of spheroids detach from the surface and form a new spheroid.
6. Glycogen staining is done by PAS reaction with or without α-amylase. The presence of glycogen is observed as positivity for an α-amylase-sensitive PAS staining.
7. The outer layer of HTC spheroids is positive for albumin and CK18 by immunochemistry. The inner layer of cells in spheroids becomes functionally inactive through the impairment of oxygen and nutrient diffusion into the spheroids.
8. The processes of making and staining these ultrathin sections require a relatively clean environment without dust.

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27

Differentiation of Embryonic Stem Cells to Retinal Cells In Vitro

Xing Zhao, Jianuo Liu, and Iqbal Ahmad

Summary

Currently, there is no effective treatment for photoreceptor degeneration, the most common cause of blindness caused by diseases like retinitis pigmentosa, age-related macular degeneration, and diabetic retinopathy. Two promising approaches include cell therapy to replace degenerating cells and neuroprotection to rescue affected cells from premature death. Determination of the potential of embryonic stem (ES) cells to differentiate into photoreceptors will provide reagents for both approaches. First, neural progenitors with retinal potential will be available in unlimited supply to test the efficacy of cell therapy; second, the controlled differentiation of ES cells into photoreceptors, in addition to providing cells to replace degenerating photoreceptors, will offer a robust in vitro model of photoreceptor differentiation for better understanding of degenerative processes and screening of neuroprotective drugs/reagents. In addition, it will allow the identification of genes (gene discovery) that play critical roles in photoreceptor differentiation and degeneration. Here, we describe the protocol to promote differentiation of the mouse ES cell-derived neural progenitors into retinal cells, specifically the rod photoreceptors.

Key Words: Differentiation; ES cells; photoreceptors; retina; stem cells.

1. Introduction

The identification and characterization of retinal stem cells/progenitors has opened the possibility of cell therapy treatment of blindness caused by retinal degeneration ***(1)***. Besides their use as transplantation reagents, these cells can serve as a model to understand the underlying mechanism of differentiation/degeneration and to test/discover cell-specific neuroprotectants. However, there are two significant barriers to the stem cell approach. First, retinal stem cells/progenitors are not available in sufficient quantity for therapeutic purposes; second, their propensity to give rise to several different retinal cell types raises the likelihood of their differentiation into undesirable glia or neurons on transplantation, which may exacerbate rather than solve problems in a highly

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ordered and laminated sensory structure like the retina. The lack of controlled differentiation also compromises their potential as a robust in vitro model for screening cell-specific neuroprotectants. In face of such roadblocks, we have examined the potential of mouse embryonic stem (ES) cells to generate retinal cell types *(2)*.

ES cells are a continuous cell line derived from preimplantation embryos. These cells were first isolated from the inner cell mass of the mouse blastocyst *(3,4)*. Subsequently, ES cells were isolated from monkeys *(5)* and humans *(6)*. What makes ES cells a robust model of early development and a reagent for cell therapy is a combination of unique properties, such as their pluripotency, stable normal karyotype, and unlimited proliferation potential. ES cells retain the pluripotent nature of the inner cell mass; when reintroduced into developing blastocysts, they participate in the formation of cell types in the chimeric mouse and contribute to tissues of all the germ layers, including gonadal tissues *(7)*.

ES cells display a remarkable capacity for continuous replication while maintaining the stable karyotype; these cells maintain normal diploid karyotype even after 140 cycles of division *(8)*. The ability of ES cells to generate virtually every cell type in the chimeric mouse suggested that these cells have an intrinsic capacity to generate specific cells provided they are exposed to appropriate developmental cues. Subsequently, it was demonstrated that ES cells can be induced to differentiate into a wide range of cell types, including neurons and glia, thus providing a valuable model system to test and study different developmental pathways *(9)*.

ES cells can be readily induced to generate neural progenitors. Currently, there are three methods of neural induction of ES cells *(10)*. Two of those require the exposure of aggregates of ES cells, called the embryoid bodies (EBs), to retinoic acid (RA) *(11)* and insulin-transferrin-selenium fibronectin (ITSFn) plus fibroblast growth factor (FGF) 2 *(12)*. An alternative method of spontaneous neural induction of ES cells has been reported that does not require the intervening steps of EB formation *(13)*. Because this method requires low-density ES cell culture and spontaneous induction, the yield of neural progenitors is relatively low. ES cell-derived neural progenitors employ the same developmental mechanism as seen in vivo for their differentiation into neurons and glia.

This notion is supported by the study of the chimeric brain resulting from the injection of ES cells into the ventricle of the developing rat *(14)*. The injected cells acquired characteristics of site-specific neurons, suggesting that ES cells possess the capacity to recapitulate the developmental program of neural stem cells. The ability of ES cells to differentiate into site-specific neurons in the chimeric brain suggested that their differentiation along specific lineages could be influenced by the environment and therefore could be amenable to controlled differentiation.

Here, we describe the protocol to promote differentiation of ES cell-derived neural progenitors into retinal cell types. This protocol works well with neural progenitors induced by either RA or ITSFn plus FGF-2. The differentiation of ES cell-derived retinal progenitors is promoted by co-culturing them with cells dissociated from embryonic retina that are known to elaborate factors that influence differentiation of retinal progenitors *(15–17)*. The efficiency of retinal differentiation, particularly along photoreceptor lineage, may be enhanced by ectopic expression of the photoreceptor regulator Crx *(18–20)*.

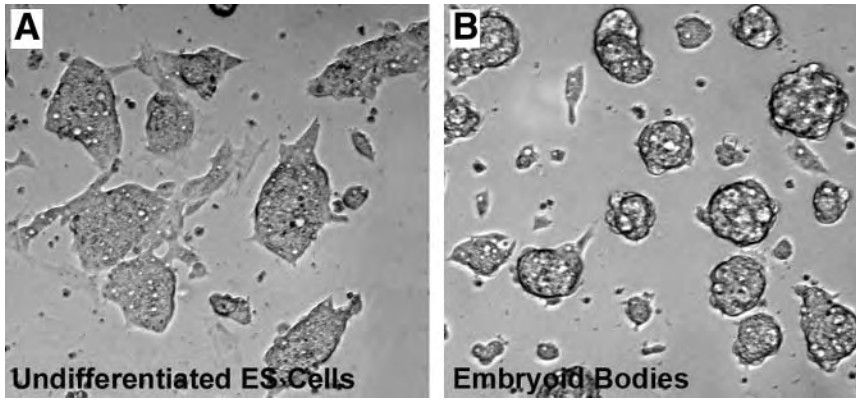

Fig. 1. Nomarski images representing (**A**) undifferentiated embryonic stem cells and (**B**) embryoid bodies. Magnification ×200.

2. Materials

2.1. Maintenance of ES Cells

Reagents are included for both the RA *(11)* and ITSFn plus FGF2 *(12)* neural induction methods.

1. Dulbecco's modified Eagle's medium (DMEM) with high glucose and pyridoxine hydrochloride and without L-glutamine and sodium pyruvate (Gibco, Grand Island, NY; cat. no. 11960-044). Store at 4°C (*see* **Note 1**).
2. Fetal bovine serum (FBS) (Hyclone, Logan, UT; cat. no. SH30070.02). Store at –20°C (heat inactivated at 56°C for 30 min) (*see* **Note 2**).
3. Newborn calf serum (Gibco, cat. no. 26010-074). Store at –20°C.
4. 200 m*M* L-glutamine (100X; Gibco, cat. no. 25030-081). Store at –20°C.
5. Nucleosides mix (100X; RA induction method): 80 mg adenosine (Sigma, St. Louis, MO; cat. no. A 4036), 85 mg guanosine (Sigma, cat. no. G6264), 73 mg cytidine (Sigma, cat. no. C4654), 73 mg uridine (Sigma, cat. no. U3003), and 24 mg thymidine (Sigma, cat. no. T1895). Dissolve in 100 mL double-distilled water (ddH_2O) at 37°C. Filter and store at 4°C. Warm at 37°C to dissolve precipitated nucleosides before use.
6. Leukemia inhibitor factor (LIF) (Chemicon, Temecula, CA; cat. no. LIF2010), 1000 U/mL working concentration. Store up to 6 mo at 4°C.
7. Mercaptoethanol 2-(ME) (100X; Fisher, Fair Lawn, NJ; cat. no. 03446-100) 10^{-4} *M* working concentration: add 7 µL 14.3 *M* 2-ME in 10 mL PBS and sterilize using 0.22-µm filter. Store up to 1 wk at 4°C.
8. 10 m*M* minimum essential medium nonessential amino acids solution (Gibco, cat. no. 11140-050) (ITSFn plus FGF-2 induction method). Store at 4°C.
9. 0.1% gelatin solution: dissolve 0.5 g gelatin (Sigma, cat. no. G-2500) in 500 mL sterilized water at 37°C. Filter-sterilize.
10. 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco, cat. no. 25300-054). Store at 4°C.
11. Phosphate-buffered saline (PBS).

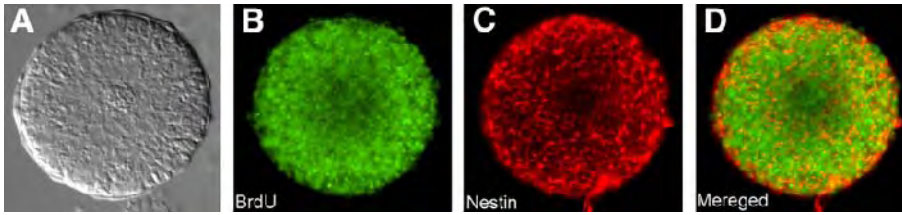

Fig. 2. Retinoic acid-induced embryoid bodies contain proliferating cells (5-bromo-2′-deoxyuridine-positive cells) **(B)** that express neuroectodermal stem cell marker nestin **(C)**. (**A**) and (**D**) represent Nomarski and merged images, respectively. Magnification ×100. (Please *see* the companion CD for the color versions of this figure.)

12. ES cell culture medium (RA induction method): 38.5 mL DMEM, 5 mL FBS, 5 mL newborn calf serum, 0.5 mL 200 m*M* L-glutamine, 0.5 mL 100X nucleosides, 50 µL LIF, and 0.5 mL 100X 2-ME. Store at 4°C.
13. ES cell culture medium (ITSFn plus FGF-2 induction method): 41 mL DMEM, 7.5 mL FBS, 0.5 mL 200 m*M* L-glutamine, 0.5 mL 10 m*M* nonessential amino acids solution, 50 µL LIF, and 0.5 mL 100X 2-ME. Store at 4°C.
14. Dimethyl sulfoxide (Fisher, cat. no. BP231-1).

2.2. Neural Induction of ES Cells

2.2.1. RA Induction Method

1. All-*trans* RA (Sigma, cat. no. R2625). Stock solution (1 m*M*): dissolve 50 mg RA in 156 mL 100% ethanol and add 8 mL distilled water. Filter and store in dark up to several months at 4°C. Working concentration: 0.5 µ*M*.
2. 5-Bromo-2′-deoxyuridine (BrdU) (Sigma, cat. no. B 5002) 1 m*M* stock: dissolve 3.07 g BrdU in 10 mL PBS at 60°C. Filter-sterilize, aliquot, and store at –20°C. Working concentration: 10 µ*M*.
3. 1 mg/mL poly-D-lysine stock (2X; Sigma, cat. no. P 0899): dissolve 10 mg poly-D-lysine in 10 mL 0.1 *M* boric acid solution (pH 8.0). Aliquot and store at –20°C. 1:1 diluted in 0.1 *M* boric acid before coating. Working concentration: 0.5 mg/mL.
4. Laminin solution (5 µg/mL) (BD Biosciences, Bedford, MA; cat. no. 354232): add 5 µg laminin in every milliliter of culture medium. Prepare fresh when needed.
5. 12-mm round glass cover slips (Fisher, cat. no. 12-545-82).

2.2.2. ITSFn Plus FGF-2 Induction Method

1. DMEM/F12 without L-glutamine and without sodium bicarbonate (Cellgro, Herndon, VA; cat. no. 90-091-PB): dissolve 11.8 g DMEM/F12 and 2.44 g $NaHCO_3$ in 1 L ddH_2O. Filter-sterilize and store at 4°C.
2. 200 m*M* L-glutamine (*see* **Subheading 2.1.**, **item 4**).
3. 5 mg/mL insulin stock (1000X; Sigma, cat. no. I 5500): dissolve 500 mg insulin in 100 mL sterile 10^{-4} *M* HCl in double-distilled water (=1 mL 0.01 *M* HCl and 99 mL sterile ddH_2O). Aliquot and store at –20°C. Working concentration: 5 µg/mL.
4. 10 mg/mL transferrin stock (200X; Sigma, cat. no. T 1428). Store at –20°C. Working concentration: 50 µg/mL.
5. 30 µ*M* sodium selenite stock (1000X; Sigma, cat. no. S 5261). Store at –20°C. Working concentration: 30 n*M*.

6. 1 mg/mL fibronectin stock (200X; Gibco, cat. no. 33016-015). Store at –20°C. Working concentration: 5 µg/mL.
7. ITSFn medium: 49 mL DMEM/F12, 0.5 mL 200 m*M* L-glutamine, 50 µL 5 mg/mL insulin, 250 µL 10 mg/mL transferrin, 50 µL 30 µ*M* sodium selenite, and 250 µL 1 mg/mL fibronectin.
8. N2 supplement stock (100X; Gibco, cat. no. 17502-048). Store at –20°C.
9. Laminin at a working concentration of 1 µg/mL (*see* **Subheading 2.2.1.**, **item 4**).
10. 25 µg/µL FGF-2 stock (Sigma, cat. no. F 0291). Aliquot and store at –20°C. Working concentration: 10 ng/mL.
11. 1 mg/mL heparin stock (Sigma, cat. no. H 3149). Store at 4°C. Working concentration: 2 µg/mL.
12. FGF-2 medium: 49 mL DMEM/F12, 0.5 mL 200 m*M* L-glutamine, 0.5 mL 100X N2 supplement, 50 µg laminin, 20 µL 25 µg/µL FGF-2, and 100 µL 1 mg/mL heparin.
13. 1 m*M* BrdU stock (*see* **Subheading 2.2.1.**, **item 2**).

2.3. Retinal Differentiation of ES Cells

1. DMEM/F12 (*see* **Subheading 2.2.2.**, **item 1**).
2. 200 m*M* L-glutamine (*see* **Subheading 2.1.**, **item 4**).
3. N2 supplement stock (100X; *see* **Subheading 2.2.2.**, **item 8**).
4. Penicillin-streptomycin (100X; Gibco, cat. no. 15140-122). Store at –20°C.
5. 25 mg/mL amphotericin B stock (1000X; Fluka, St. Louis, MO; cat. no. 10041). Store at –20°C. Working concentration: 25 µg/mL.
6. FBS (Cellgro, cat. no. 35-011-CV).
7. Serum-free retinal culture medium (RCM): 48.5 mL DMEM/F12, 0.5 mL 200 m*M* L-glutamine, 0.5 mL 100X N2 supplement, 0.5 mL 100X penicillin-streptomycin, and 50 µL 25 mg/mL amphotericin B.
8. HBSS (Cellgro, cat. no. 21-020-CV).
9. HBSS without calcium and magnesium (Cellgro, cat. no. 21-021-CV).
10. 0.25% trypsin (Gibco, cat. no. 15050-065).
11. Deoxyribonuclease (DNase) 1 (Sigma, cat. no. D 4513) stock (4000 U/mL).
12. 100 m*M* EDTA.
13. Dissociation solution: 3 mL HBSS (Ca^{++} and Mg^{++} free), 2 mL 0.25% trypsin, 100 µL DNase 1 stock, and 50 µL 100 m*M* EDTA.
14. 0.4 µm Millicell CM membrane (Millipore, Billerica, MA; cat. no. PICM03050).

2.3.1. Retinal Differentiation by Ectopic Expression of Crx

1. Crx cDNA (***19***).
2. pIRES2-EGFP (enhanced green fluorescent protein) vector (Clontech, Palo Alto, CA; cat. no. 6029-1).
3. Plasmid maxikit (Qiagen, Valencia, CA; cat. no. 12163).
4. Effectene transfection kit (Qiagen, cat. no. 301425).
5. BTX Electro Square Porator ECM 830 (BTX, San Diego, CA).
6. 4-mm gap cuvet (BTX, model no. 640).

2.4. Immunocytochemical Analysis

1. PBS.
2. 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA; cat. no. 19208): add 8 g paraformaldehyde to 100 mL ddH_2O; add several drops 10 *N* NaOH while

Table 1
List of Primary Antibodies for Immunofluorescence

Antibody	Specific cell marker	Species	Dilution	Source/reference
Notch1	Stem cells	Rat	1:1	*21*
Nestin	Neural stem cells	Mouse	1:4	DSHB, Iowa City, IA
Pax6	Retinal stem cells	Rabbit	1:1000	Covance, Berkeley, CA; cat. no. PRB 2789
Chx10	Retinal stem cells	Rabbit	1:500	*22*
PKC	Bipolar cells	Rabbit	1:1000	Sigma, cat. no. P 4334
mGluR6	Bipolar cells	Mouse	1:250	*23*
Rx	Rod photoreceptors	Mouse	1:250	*24*
Crx	Rod photoreceptors	Rabbit	1:200	*25*
Nrl	Rod photoreceptors	Rabbit	1:200	*26*
Opsin	Rod photoreceptors	Mouse	1:5000	*27*
Rhodopsin kinase	Rod photoreceptors	Mouse	1:500	Affinity BioReagents, Golden, CO; cat. no. MA1 720
Arrestin	Rod photoreceptors	Mouse	1:100	*28*
Peripherin	Rod photoreceptors	Rat	1:15	*29*
IRBP	Rod photoreceptors	Mouse	1:100	*30*
Syntaxin	Amacrine cells	Mouse	1:100	*27*
BrdU	Dividing cells	Rat	1:100	ACCU, Westbury, NY; cat. no. OBT0030

stirring until mostly dissolved. Add 66.67 mL 3X PBS; adjust to pH 7.2. Bring volume to 200 mL with double-distilled water, filter-sterilize, and store at 4°C.

3. Normal goat serum (NGS) (Sigma, cat. no. G 9023): aliquot and store at –20°C.
4. 10% Triton X-100 (Fisher, cat. no. BP151-100) in PBS.
5. No Triton blocking solution: 9.5 mL 1X PBS, and 0.5 mL NGS. 0.2% Triton blocking solution: 9.3 mL 1X PBS, 0.5 mL NGS, and 0.2 mL 10% Triton. 0.4% Triton blocking solution: 9.1 mL 1X PBS, 0.5 mL NGS, and 0.4 mL 10% Triton (*see* **Note 3**).
6. 2 *N* HCl (Fisher, cat. no. A144-500): add 43 mL concentrated HCl slowly to 228.45 mL ddH_2O while mixing under the fume hood.
7. 0.1 *M* boric acid (Fisher, cat. no. BP168-500): dissolve 6.183 g boric acid in PBS, adjust to pH 8.3, then bring volume to 1 L. Filter-sterilize.
8. Fluromount-G (Southern Biotechnology Associates, Birmingham, AL; cat. no. 0100-01). Store at 4°C.
9. Primary antibodies used to determine cell-type specificity by immunofluorescence analyses (*see* **Table 1**; **Note 4**).
10. Secondary antibodies used for immunofluorescence analyses (1:400 dilution, Jackson Immuno Research Laboratories, West Grove, PA) (*see* **Table 2**).

Table 2
List of Secondary Antibodies for Immunofluorescence

Antibody	Cat. no.
Cy3-conjugated goat antirat IgG	112-165-102
FITC-conjugated goat antirat IgG	112-0950167
Cy3-conjugated goat antimouse IgG	115-165-100
Cy3-conjugated goat antirabbit IgG	111-165-144
Cy3-conjugated donkey antigoat IgG	705-165-147

2.5. Reverse Transcriptase Polymerase Chain Reaction Analysis

2.5.1. RNA Extraction

1. 2.5-mL glass homogenizer (Wheaton, Millville, NJ).
2. Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH; cat. no. TR 118). Store at 4°C.
3. Chloroform (Fisher, cat. no. BP1145-1).
4. Isopropanol (Acros, Belgium; cat. no. 327270010).
5. 75% ethanol (Aldrich, St. Louis, MO; cat. no. 45984-4).
6. DNase treatment and removal reagents (Ambion, Austin, TX; cat. no. 1906). Store at –20°C.

2.5.2. Complementary DNA Synthesis

1. SuperScript RNase H^- reverse transcriptase (RT) (Invitrogen, Carlsbad, CA; cat. no. 18053-017). Store at –20°C. This set also contains 5X first-strand buffer and 100 m*M* dithiothreitol.
2. Recombinase RNasin ribonuclease (RNase) inhibitor (Promega, Madison, WI; cat. no. N2511). Store at –20°C.
3. 100 nmol/µL random hexamers pDN6 (Amersham, Piscataway, NJ; cat. no. 27-2166-01). Store at –20°C.
4. Polymerase chain reaction (PCR)-grade 100 m*M* dNTP set (Invitrogen, cat. no. 10297-018). Store at –20°C.

2.5.3. PCR Analysis and Gel Electrophoresis

1. *Taq* DNA polymerase (Fermentas, Hanover, MD; cat. no. EP0402). Store at –20°C. This set also contains 10X PCR buffer and 25 m*M* $MgCl_2$.
2. 100 m*M* dNTP (*see* **Subheading 2.5.2., item 4**).
3. Gene Ruler 100-bp DNA Ladder Plus (Fermentas, cat. no. SM0322). Store at –20°C.
4. 6X loading dye solution (MBI Fermentas, cat. no. R0611). Store at –20°C.
5. Agarose (Invitrogen, cat. no.15510-019).
6. 10X TAE buffer: dissolve 48.4 g Tris-base (Invitrogen, cat. no. 15504-020) in 900 mL ddH_2O. Add 11.42 mL acetic acid (Fisher, cat. no. BP1185-500) and 20 mL 0.5 *M* EDTA (Acros, cat. no. 118432500); bring volume to 1 L. Autoclave and store.
7. Ethidium bromide stock (10 mg/mL): dissolve 10-mg tablet (Sigma, cat. no. E4391) in 1 mL ddH_2O. Final concentration is 0.2 µg/mL.
8. Primers used for RT-PCR analyses (*see* **Table 3**).

Table 3
List of Primers for Specific Gene Amplification

Genes	Primer sequences	Specificity	Annealing temperature	Size (bp)	Accession number
Chx10	Forward TCCGATTCCGAAG ATGTTTCC	Bipolar cells	58	350	L34808
	Reverse GACTTGAGGATAGA CTCTGGCAGG				
mGluR6	Forward TCCGGTGGCCAGT CAGATGAT	Bipolar cells	56	426	D13963
	Reverse CACTTCGTGGGGGT CGCCTGT				
Crx	Forward CCTCACTATTCGGT CAATGCC	Rods	58	346	NM021855
	Reverse ATGTGCCTGCCTTC CTCTTC				
Nrl	Forward TTTGGAGGTGGCT GGGTAGATG	Rods	50	114	NM008736
	Reverse ACGATGCTCAGAAG TTTGGGG				
Rhodopsin	Forward CATGCAGTGTTCAT GTGGGA	Rods	64	422	NM033441
	Reverse AGCAGAGGCTGGTG AGCATG				
Rhodopsin	Forward GCTGAACAAGAA GCGGCTGAAG	Rods	56	238	U63971
Kinase	Reverse TGCTGTGTAGTAGA TGGCTCGTGG				
Arrestin	Forward GCTCGTGAAGGGG AAGAAAGTG	Rods	58	324	M60737
	Reverse TCTCTGATGTCTGT GGCAAATGC				
IRBP	Forward AGGGAACTTTGG ACACACT	Rods	56	336	X56159
	Reverse CTGGAGTATCTCC TTGGC				
Syntaxin	Forward AAGAGCATCGAG CAGAGCATC	Amacrine cells	60	342	AF217191
	Reverse CATGGCCATGTCT CATGAACA				
β-*actin*	Forward GTGGGGCGCCCC AGGCACCA	Housekeeping	50	548	BC004251
	Reverse CTCCTTAATGTCA CGCACGATTTC				

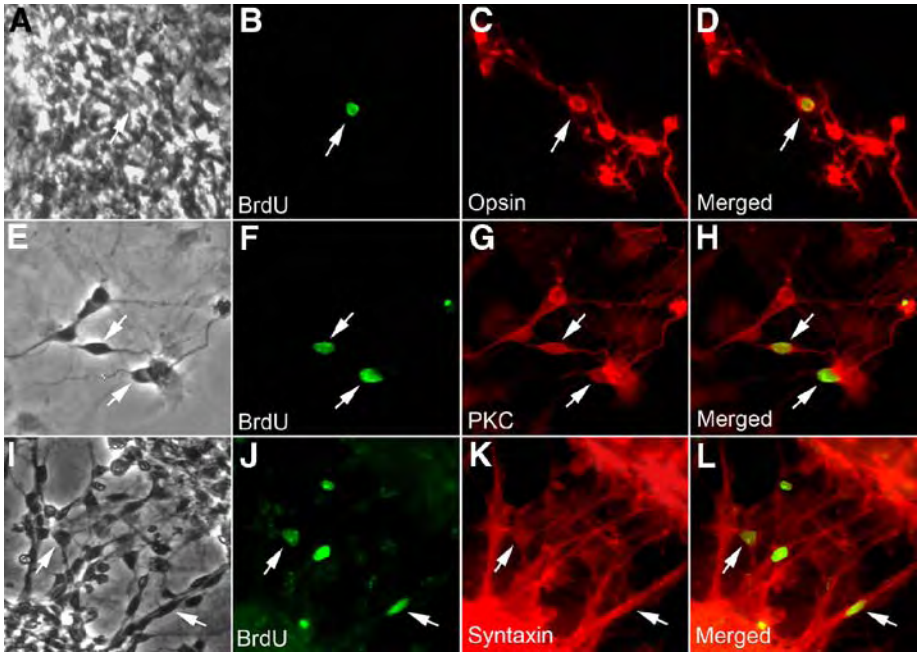

Fig. 3. Retinoic acid-induced embryonic stem cells (5-bromo-2′-deoxyuridine-tagged cells) **(B**,**F**,**J)** differentiate along retinal lineage as demonstrated by their expressions of opsin **(C)**, a rod photoreceptor marker; PKC **(G)**, a bipolar cell marker; and syntaxin **(K)**, an amacrine cell marker. (**A**, **E**, and **I**) represent Nomarski images and (**D**, **H**, and **L**) represent merged images. Magnification ×200. (Please *see* the companion CD for the color versions of this figure.)

3. Methods

3.1. Maintenance of D3 ES Cells

3.1.1. Gelatin Coating of Flasks

All dishes, flasks, and plates are gelatinized before use.

1. Add 5 mL 0.1% gelatin solution into a T-25 flask to cover the bottom surface.
2. Coat the flask for at least 30 min at room temperature inside the hood.
3. Aspirate the gelatin solution completely just before use (*see* **Note 5**).

3.1.2. Thawing of ES Cells

1. Thaw a frozen vial of cells in 37°C water bath with gentle agitation.
2. Transfer the cell suspension into a 15-mL centrifuge tube containing 5 mL 37°C prewarmed ES cell culture medium.
3. Centrifuge at 1500*g* for 5 min at 8°C. Resuspend cells in fresh ES cell culture medium.
4. Plate 2×10^6 cells/T-25 flask in 5 mL ES cell culture medium.
5. Feed cells with 5 mL fresh medium the next day. Cells are nearly confluent in 2 d.

3.1.3. Passage of ES Cells

1. Aspirate medium and wash cells once with 5 mL prewarmed PBS.
2. To lift cells from the flask, add 1 mL 0.05% trypsin-EDTA. Incubate at 37°C for 10 min.

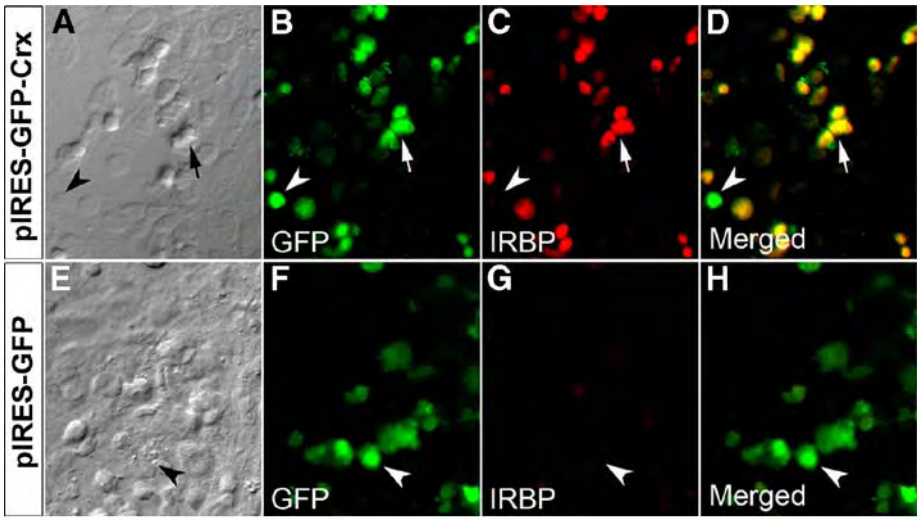

Fig. 4. Evaluation of the expression of photoreceptor-specific marker IRBP in cells transfected with Crx-expressing vector. A selected field of cells showing cells transfected with internal ribosome entry site green fluorescent protein (IRES2-GFP)-Crx **(B)**, expressing IRBP (**C**,**D**, arrow). Arrowhead indicates that not all GFP-positive cells are IRBP positive. IRBP immunoreactivity was undetectable **(G**,**H)** in cells transfected with control vector, IRES2-GFP **(F)**. (**A**,**E**) represent Nomarski images and (**D**,**H**) represent merged images. Magnification ×200. (Please *see* the companion CD for the color version of this figure.)

3. Add 5 mL ES cell culture medium to quench trypsin. Collect cell suspension into a 15-mL centrifuge tube and triturate about 10 times to obtain single-cell suspension.
4. Centrifuge at 1500*g* for 5 min at 8°C. Resuspend cells in ES cell culture medium and plate at 2×10^6/T-25 flask. Cell number will increase to around 18×10^6 in 2 d.
5. Cells are passaged every second day (*see* **Note 6**).

3.1.4. Freezing ES cells

1. Resuspend 4×10^6 cells in 0.5 mL ice-cold ES culture medium and keep on ice.
2. Add 0.5 mL 2X freezing medium (20% dimethyl sulfoxide plus 80% ES cell culture medium kept on ice); mix well. Freeze cells at –80°C overnight. Transfer vials to liquid nitrogen the next day.

3.2. Neural Induction of ES Cells

3.2.1. RA Induction Method

1. Prepare ES cell culture medium without LIF and 2-ME. Warm to 37°C.
2. Trypsinize cells (*see* **Subheading 3.1.3., step 3**).
3. Add 5 mL ES cell culture medium without LIF and 2-ME to quench trypsin. Collect cell suspension in a 15-mL tube and triturate 10 times.
4. Spin down cells at 1500*g* for 5 min. Resuspend cells in incomplete culture medium.
5. Plate 4–5 $\times 10^6$ cells in 10 mL incomplete culture medium in a 100-mm nonadhesive Petri dish. Cell aggregates (EBs) are observed a day later.

6. The medium is changed by sedimentation of EBs. The suspension of EBs is transferred to a 15-mL tube. Allow the aggregates to settle for 10 min at room temperature. Aspirate the medium gently and replace with fresh medium.
7. After 4 d, the medium is changed by sedimentation with fresh medium containing all-*trans* RA (0.5 µ*M*).
8. The culture is continued for 4 d more. BrdU (10 µ*M*) is added to the final 24 h of culturing to label dividing cells.
9. Coat 12-mm round glass cover slips by covering the surface with 80 µL poly-D-lysine solution (0.5 mg/mL) at room temperature overnight. The next day, transfer cover slips to 24-well plate, wash three times with PBS, and air-dry for 10 min at room temperature. Prepare 5 mg/mL laminin solution in culture medium; coat cover slips with laminin at room temperature for 1 h. Aspirate the solution completely before use.
10. Transfer an aliquot of EBs to precoated glass cover slips the night before fixation (*see* **Subheading 3.2.2.**, **step 6**). Cover slips can be stored in PBS at 4°C for up to 2 wk after fixation until immunocytochemical analysis.
11. Freeze an aliquot of EBs for RT-PCR analysis.
12. The culture medium is changed every other day.

3.2.2. ITSFn plus FGF-2 Induction Method

1. Generate EBs as described in **Subheading 3.2.1.**, in nonadhesive dishes for 4 d.
2. Transfer EB suspension to 100-mm adhesive tissue culture surface dish and let EBs attach for 24 h.
3. Aspirate the medium completely and wash twice with PBS.
4. Culture EBs in 10 mL ITSFn medium for 6–10 d.
5. Dissociate cells in 0.05% trypsin-EDTA, plate ES cells at a density of 1.5–2 × 10^5/cm^2 onto poly-D-lysine plus laminin precoated cover slips/plates, and culture in FGF-2 medium for 6 d.
6. Fix cells in 4% paraformaldehyde for 15 min at 4°C for immunocytochemical analysis and freeze an aliquot for RT-PCR analysis.

3.3. Retinal Differentiation

1. Collect ES cells following neural induction in 15-mL centrifuge tubes and spin down at 1500*g* for 5 min.
2. Resuspend cell pellet in 5 mL 0.05% trypsin-EDTA and incubate at 37°C for 10 min.
3. Neutralize trypsin with 500 µL FBS, triturate 20 times, and add 5 mL RCM.
4. Centrifuge at 1500*g* for 5 min and resuspend cells in RCM plus 1% FBS.
5. Plate cells on poly-D-lysine and laminin-coated glass cover slips at a density of 2 × 10^5/well (24-well plate) or 1 × 10^6/well (6-well plate).
6. Retinas from postnatal (PN) d 1 rats are dissected out in HBSS as previously described ***(15)*** and transferred to a 15-mL centrifuge tube.
7. Incubate the retina in 5 mL dissociation solution at 37°C for 10 min.
8. Add 200 µL FBS to quench trypsin and triturate 15 times with a 5-mL pipet.
9. Add 5 mL RCM medium and centrifuge at 1500*g* for 5 min.
10. Resuspend retinal cells in RCM plus 1% FBS.
11. Induced ES cells are co-cultured with PN1 retinal cells (2 × 10^5/well, 24-well plate; 1 × 10^6/well, 6-well plate) in RCM plus 1% FBS for 5–15 d.
12. The medium is changed every other day. Cells are fixed in paraformaldehyde for immunocytochemical analyses (**Fig. 5**). ES cells and PN1 retinal cells are co-cultured across a 0.4-µm Millicell CM membrane in six-well plates for RT-PCR analysis.

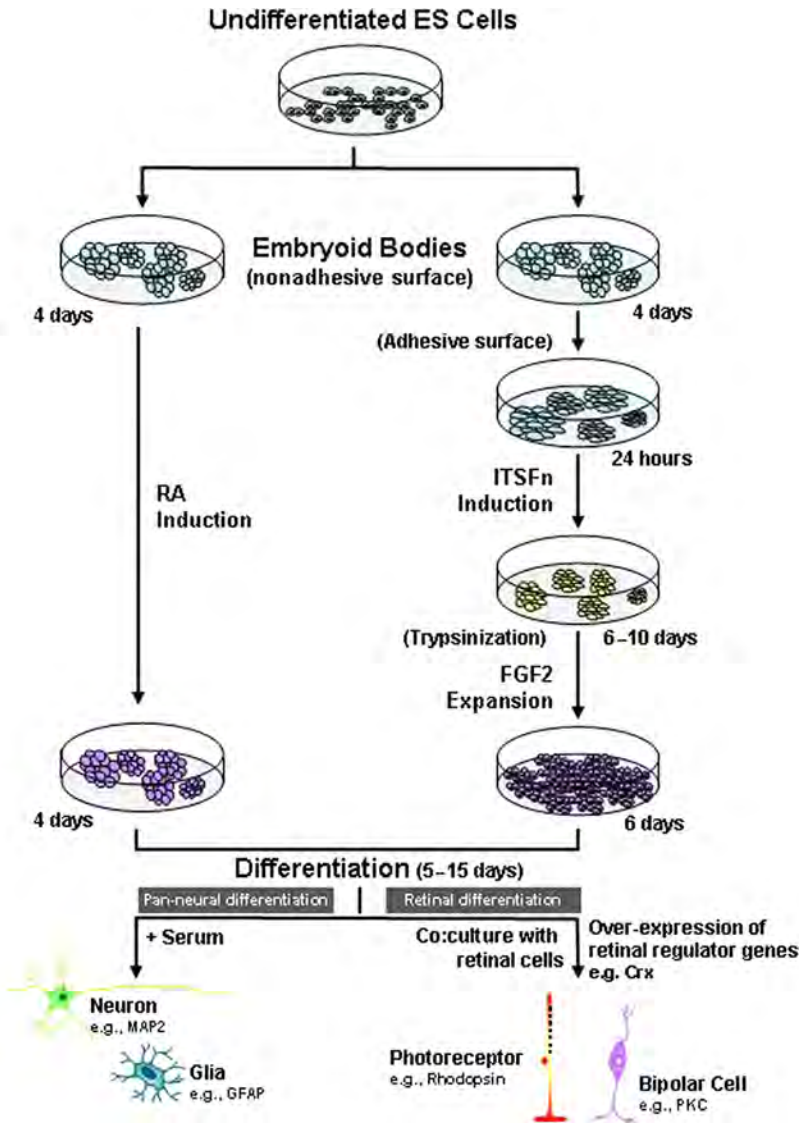

Fig. 5. Overview of retinoic acid- and insulin-transferrin-selenium fibronectin plus fibroblast growth factor-2-based neural induction and retinal differentiation methods. (Please *see* the companion CD for the color version of this figure.)

3.3.1. Retinal Differentiation by Ectopic Expression of Crx

1. Crx cDNA is cloned into pIRES2-EGFP vector, which expresses green fluorescent protein (GFP) through internal ribosome entry site (IRES) by the standard method ***(31)***.
2. Recombinant plasmid is amplified using the Qiagen plasmid maxikit following the manufacturer's protocol.

3. Cultured ES cells have been used for neural induction (*see* **Subheading 3.2.**). The night before transfection, ES cells were trypsinized and plated in a poly-D-lysine precoated six-well plate at a density of 1×10^6/well. Cells should be more than 80% confluent the next morning.
4. Transfection is done based on the manufacturer's instruction. Briefly, 0.4 µg DNA plasmid is mixed with 3.2 µL enhancer in buffer EC to make a final volume of 100 µL and incubated for 3 min at room temperature. Add 10 µL Effectene reagent to the mixture and incubate for 8 min. The mixture is then mixed with 600 µL culture medium and added directly to the cells. Transfection efficiency is evaluated in 48 h.
5. Alternatively, plasmids can be electroporated as follows: 20 µg DNA plasmid is added to 5×10^6 dissociated ES cells, and the total volume is brought to 50 µL. The mixture is then transferred to 4-mm cuvet and electroporated at 100 V for 50 ms with five pulses at 950-ms intervals. The cells are immediately resuspended in 6 mL culture medium and plated in a six-well plate (1 mL/well). Transfection efficiency is evaluated in 48 h.

3.4. Immunocytochemical Analysis

1. Paraformaldehyde fixed cells are washed once with PBS before incubation in an appropriate blocking solution at room temperature for 30 min (*see* **Subheading 2.4.**, **item 5**).
2. Aspirate blocking solution and add primary antibody solution (appropriately diluted in the blocking solution).
3. Incubate at 4°C overnight or at room temperature for 2 h.
4. Wash cover slips three times with PBS for 10 min each.
5. Add secondary antibody solution (diluted 1:400 in blocking solution) and incubate in the dark at room temperature for at least 1 h.
6. Aspirate secondary antibody and wash three times with PBS for 10 min each.
7. Mount the cover slips on glass slides with Fluoromount-G and store in the dark at 4°C until microscopic examination.

3.4.1. Detection of BrdU Incorporation

After the first immunocytochemical analysis to localize a particular cell-specific antigen, cells incorporating BrdU are detected as follows:

1. Cells are incubated in 2 *N* HCl at 37°C for 45 min to denature DNA.
2. HCl is washed out with PBS and neutralized by incubating cells in 0.1 *M* boric acid at room temperature for 10 min.
3. Cells are washed three times with PBS for 10 min each (*see* **Note 7**).
4. Immunocytochemical analysis is carried out to detect BrdU using anti-BrdU antibody as described in **Subheading 3.4.**, **steps 2–7**.

3.5. RT-PCR Analysis

3.5.1. RNA Extraction

1. Collect ES cells by centrifuging at 1000*g* for 3 min, add 1 mL Tri-reagent, and homogenize using a 2.5-mL glass homogenizer.
2. Incubate at room temperature for 5 min.
3. Add 200 µL chloroform; mix vigorously for 10 s.
4. Incubate at room temperature for 15 min.
5. Spin at 12,000*g* for 15 min at 4°C.
6. Transfer aqueous phase to a new tube; add 0.5 mL isopropanol.
7. Store at –20°C overnight (minimum 1 h).

8. Spin at 12,000*g* for 8 min at 4°C.
9. Wash the pellet with 75% ethanol.
10. Spin at 7500*g* for 5 min at 4°C.
11. Dry pellet and dissolve in a minimum amount of water (10–50 µL).

3.5.2. DNase Digestion

To remove residual DNA from RNA samples:

1. Add 0.1 vol 10X DNase 1 buffer and 1 µL DNase 1 to RNA sample, mix, and incubate at 37°C for 20–30 min.
2. Neutralize the enzyme by adding 0.1 vol (or 5 µL, whichever is greater) of the inactivation reagent. Mix and incubate at room temperature for 2 min.
3. Centrifuge the solution at 10,000*g* for 1 min to pellet the DNase inactivation reagent.
4. Transfer the supernatant, containing RNA, to a new tube and measure the RNA concentration.

3.5.3. cDNA Synthesis

1. The master mix for cDNA synthesis is prepared as follows: 10 µL 5X first-strand buffer, 5 µL pDN6, 5 µL 100 m*M* dithiothreitol, 2.5 µL 10 m*M* dNTP, 1 µL RNAsin, and 2 µL SuperScript.
2. Add RNA (2–5 µg) and bring the total volume to 50 µL with diethylpyrocarbonate-treated water.
3. cDNA synthesis is carried out on the Robocycler PCR machine using the following step cycle: 37°C for 60 min, 42°C for 30 min, and 70°C for 10 min. cDNA is stored at –20°C.

3.5.4. PCR Analysis

1. The master mix for PCR amplification is made as follows: 10 µL 10X PCR buffer, 2 µL 25 m*M* $MgCl_2$, 2 µL 10 m*M* dNTP, 1 µL gene-specific primer-forward, 1 µL gene-specific primer-reverse, and 0.5 µL *Taq* DNA polymerase.
2. Add cDNA (1–2 µL) and bring the total volume to 50 µL with double-distilled water.
3. The PCR amplification is carried out on the Robocycler PCR machine using the following step cycles: 94°C for 3 min, 94°C for 30 s, 56°C (the reannealing temperature will depend on specific primer sets; refer to **Table 3** for correct temperature) for 40 s, 72°C for 45 s (for 25 cycles), 72°C for 5 min.
4. Analyze PCR products by electrophoresis using 2% agarose gel in 1X TAE buffer.

4. Notes

1. Because L-glutamine is quickly degraded, it should be added fresh to the culture medium.
2. The quality of serum is critical for successful culturing of ES cells. Several batches of serum should be tested to select one that promotes optimal growth. Serum should be heat inactivated at 56°C for 30 min and stored at –20°C.
3. The concentration of Triton in the blocking solution differs depending on whether the antigen is located on the cell surface (0%) or cytoplasmid (0.2%) or is nuclear (0.4%).
4. Some of the markers that identify specific cell types in the retina are also expressed elsewhere in the brain. For example, syntaxin, the marker for amacrine cells, and mGluR6, the marker for bipolar cells, are not expressed exclusively in the retina. Therefore, it is extremely important that multiple markers for specific retinal cell types are screened for the examination of retinal differentiation of ES cells. For that reason, the examination of the differentiation of ES cells into photoreceptors is preferable because photoreceptor-specific markers such as rhodopsin and rhodopsin kinase are retina specific.
5. Do not let the coated surface dry.

6. ES cells have to be plated as single cells; otherwise, ES cells will differentiate even in the presence of LIF. A long-term culture of ES cells can accumulate genetic mutations and chromosome abnormalities. Therefore, in vitro passaging of ES cells should not be allowed for more than 4 wk.
7. Minimize the exposure to light, which can quench the fluorescence of the secondary antibody.

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Derivation and Characterization of Lentoid Bodies and Retinal Pigment Epithelial Cells From Monkey Embryonic Stem Cells In Vitro

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Summary

For cell replacement therapies of retinal diseases, the most-needed cells are retinal pigment epithelial cells and photoreceptor cells; lens cells are needed for replacement of the cataract. Stromal cell-derived inducing activity induces the differentiation of mouse embryonic stem (ES) cells into neural cells, including midbrain tyrosine hydroxylase-positive dopaminergic neurons, and ocular cells; the method also works with primate ES cells. We describe the methods to induce retinal pigment epithelial and lens cells from monkey ES cells using stromal cell-derived inducing activity and show the characteristics of those cells in vitro and in vivo.

Key Words: Lentoid bodies; monkey ES cells; RCS rat; RPE cells.

1. Introduction

Embryonic stem (ES) cells retain significant developmental potential and replicative capability and are expected to alleviate the problem of the shortage of donor cells for replacement therapy. However, their differentiation is poorly controlled, and the purification of differentiated cells is not sufficient so far. ES cells may produce various ocular cells spontaneously; however, we need to seek the most effective methods to induce them and purify the population. Furthermore, the characteristics of ES cells considerably differ between rodents and primates; for example, primate ES cells cannot maintain immaturity without feeder cells; rodent ES cells can. Therefore, if cell replacement therapy for ocular cells is to be achieved, then we need to induce them from primates, especially human ES cells.

For cell replacement therapies of retinal diseases, the most-needed cells are retinal pigment epithelial (RPE) cells and photoreceptor cells; lens cells are needed for replacement of the cataract. The retinal pigment epithelium is a single layer of cells derived from optic vesicle that was formed from invagination of the neural tube.

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Although the origin is the same as neural retina, RPE cells are highly specialized cells located in the outside of the neural retina. They are adjacent to photoreceptor cells and have critical functions to maintain the photoreceptors. These functions include the formation of the blood-retinal barrier, regeneration of visual pigments, and phagocytosis of outer segments of photoreceptors. Dysfunction of the RPE cells causes photoreceptor degeneration, which is observed in ocular diseases such as age-related macular degeneration and some forms of retinitis pigmentosa. Lens cells, on the other hand, are derived from surface ectoderm, developed from the invagination of the lens placode into the concavity of the optic cup. The lens is a transparent organ functioning in the refraction, accommodation, and absorption of ultraviolet light. Accumulation of aggregated proteins caused by various stresses such as heat, oxidation, and exposure to heavy metal causes opacity in the lens (cataract).

Stromal cell-derived inducing activity (SDIA) induces the differentiation of mouse ES cells into neural cells, including midbrain tyrosine hydroxylase-positive dopaminergic neurons ***(1)***, and ocular cells ***(2)***; the method also works with primate ES cells ***(3)***. In this chapter, we describe the most effective methods that we have now to induce RPE and lens cells from monkey ES cells using SDIA.

2. Materials

2.1. Tissue Culture

2.1.1. Cell Lines

1. Cynomolgus monkey ES cells (Asahi Techno Glass, Chiba, Japan; cat. no. CMES-001).
2. Mouse embryonic fibroblasts (STO) (Riken BioResource Center, Tsukuba, Japan; cat. no. RCB0536).
3. MC3T3-G2/PA6 cells (Riken BioResource Center, cat. no. RCB1127).

2.1.2. Medium for STO Cells

1. Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO; cat. no. D-6429).
2. Fetal bovine serum (FBS) (HyClone, Logan, UT; cat. no. AJF10577).
3. Penicillin-streptomycin (pen-strep) (Gibco, Carlsbad, CA; cat. no. 15140-148).
4. 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco, cat. no. 15050-065).
5. PBS(–) (Gibco, cat. no. 12388-013).
6. 0.5 *M* EDTA (Gibco, cat. no. 11267-028).
7. Mitomycin C (Wako, Osaka, Japan; cat. no. 134-07911) (*see* **Note 1**).

2.1.3. Medium for PA6 Cells

1. Minimum essential medium α-(MEM) (Gibco, cat. no. 12571-063).
2. FBS (HyClone, cat. no. AJF10577).
3. Pen-strep (Gibco, cat. no. 15140-148).
4. 0.05% trypsin 1m*M* EDTA (Gibco, cat. no. 25300-054).
5. PBS(–) (Gibco, cat. no. 12388-013).

2.1.4. Medium for Monkey ES Cells

1. DMEM/F12 (Sigma, cat. no. D-6421) (*see* **Note 2**).
2. Nonessential amino acids (NEAA) (Gibco, cat. no. 11140-050).
3. L-Glutamine (Sigma, cat. no. G-2150).

4. 20% Knockout serum replacement (KSR) (Gibco, cat. no. 10828-028).
5. 2 m*M* 2-mercaptoethanol (Sigma, cat. no. M-7522).
6. 10^3 U/mL leukemia inhibitory factor (LIF) (Chemicon, Temecula, CA; cat. no. ESG1107).
7. 0.5 *M* NaOH (Wako, cat. no. 199-02185).
8. Pen-strep (Gibco, cat. no. 15140-122).
9. Basic fibroblast growth factor (bFGF) (Upstate Biotechnology, Charlottesville, VA; cat. no. 46-536).
10. Maintenance medium: DMEM/F12 supplemented with 0.1 m*M* 2-mercaptoethanol, 1000 U/mL LIF, 20% KSR, 0.1 m*M* NEAA, and 4 or 8 ng/mL bFGF. Combine 200 mL DMEM/F12 with 2 µL 2-mercaptoethanol, 25 µL LIF, 50 mL KSR, 2 mL NEAA, 2.5 mL L-glutamine, and 2.5 mL NaOH. Add 4 or 8 ng/mL bFGF just before use.

2.1.5. Subculturing of ES Cells

1. 0.25% trypsin-EDTA (Gibco, cat. no. 15050-065).
2. 20% KSR (Gibco, cat. no. 10828-028).
3. PBS (Gibco, cat. no. 10010-023).
4. 100 m*M* $CaCl_2$ (Wako, cat. no. 597-02085).
5. 2 m*M* 2-mercaptoethanol (Sigma, cat. no. M-7522).

2.1.6. Induction of Differentiation of ES Cells

1. Glasgow minimal essential medium (GMEM) (Gibco, cat. no. 11710-035).
2. NEAA (Gibco, cat. no. 11140-050).
3. Pyruvate (Sigma, cat. no. S-8636).
4. KSR (Gibco, cat. no. 10828-028).
5. Pen-strep (Gibco, cat. no. 15140-122).
6. Differentiation medium: GMEM medium supplemented with 10% KSR, 1 m*M* pyruvate, 0.1 m*M* NEAA, and 0.1 m*M* 2-mercaptoethanol. Combine 500 mL GMEM with 27 mL KSR, 5 mL pyruvate, 5 mL NEAA, and 0.5 mL 0.1 *M* 2-mercaptoethanol.

2.1.7. Medium for RPE Cells

1. DMEM (Gibco, cat. no. 11995-065).
2. FBS (HyClone, cat. no. AJF10577).
3. Pen-strep (Gibco, cat. no. 15140-122).
4. Matrigel (BD Biosciences, Franklin Lakes, NJ; cat. no. 354234): dilute 1:20 to coat dishes.
5. Collagen-coated dishes (Asahi Techno Glass, cat. no. 4020-010).

2.1.8. Gelatin-Coated Dishes

Gelatin (Sigma, cat. no. G-2625): for coating, prepare a 0.1% gelatin solution in distilled water and sterilize (*see* **Note 3**).

2.2. Immunofluorescence

1. 4% paraformaldehyde in 0.1 *M* phosphate buffer.
2. PBS (Gibco, cat. no. 10010-023).
3. Humidified chamber (*see* **Note 4**).
4. Glycerol.
5. 0.1 *M* phosphate buffer (PB) containing 0.005% saponin (0.1 *M* PB-saponin; Merck, Darmstadt, Germany; cat. no. 1.08603.1000).
6. Skim milk (Dainihon-Seiyaku, Osaka, Japan; cat. no. 232100): prepare a 20% solution in 0.1 *M* PB-saponin.

7. Laser scanning confocal microscope (Leica, Wetzlar, Germany).
8. Rabbit polyclonal anti-ZO-1 antibody (1:50; Zymed, San Francisco, CA; cat. no. 61-7300); rabbit polyclonal anti-α crystallin (1:1000; Stressgen, Victoria, Canada; cat. no. SPA-221); and anti-Pax6 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA; cat. no. SC-11357) were used as the primary antibodies. Mouse monoclonal anti-rhodopsin antibody (1:2000; Sigma-Aldrich, cat. no. R5403). Nuclei were stained with Sytox blue (1:500 in distilled water; Molecular Probes, Eugene, OR; cat. no. S11348), and the specimens were observed and photographed with a laser scanning confocal microscope (Leica, Heidelberg, Germany; model no. TCS SP2).
9. Fluorescein-conjugated donkey anti-rabbit immunoglobulin (1:100; Amersham, Buckinghamshire, UK).

2.3. Reverse Transcriptase Polymerase Chain Reaction

1. Thermal cycler (ABI, Foster City, CA; model no. Gene Amp 9700).
2. Agarose gel apparatus and reagents.
3. Trizol reagent (Gibco BRL, cat. no. 15596-026).
4. Cell scrapers (Costar, Cambridge, Mass.; cat. no. 3010).
5. 17 × 100 mm polypropylene sterile culture tubes (Fisher, Atlanta, GA; cat. no. 14-956-1J).
6. First-strand complementary DNA synthesis kit (Amersham Biosciences, Piscataway, NJ; cat. no. 27-9261-01).
7. 25 m*M* $MgCl_2$.
8. 10X PCR buffer II (ABI Roche, cat. no. N8080010).
9. 100 m*M* dNTP set (Gibco BRL, cat. no. 10297-018).
10. *Taq* DNA polymerase (5 U/μL; ABI Roche, cat. no. N8080155).
11. Diethylpyrocarbonate (DEPC)-H_2O.
12. 10X deoxyribonuclease buffer: 400 m*M* Tris-HCl (pH 7.5), 80 m*M* $MgCl_2$, 50 m*M* dithiothreitol (Takara, Shiga, Japan; cat. no. 2215A).
13. 10 m*M* dNTPs (for reverse transcription): dilute stock dNTPs 1:10 with DEPC-H_2O.
14. 2.5 m*M* dNTPs (for PCR): combine 250 μL of each stock dNTP (100 m*M*) into a sterile microtube and mix (25 m*M*). Aliquot 100 μL into each of 10 tubes and add 900 μL sterile water and mix (2.5 m*M*).

2.4. Western Blot Analysis

1. Laemmli sample buffer: 62.5 m*M* Tris-HCl at pH 6.8, 25% glycerol, 2% sodium dodecyl sulfate, 0.01% bromophenol blue (Bio-Rad, Hercules, CA; cat. no. 161-0737).
2. 5 m*M* 2-mercaptoethanol (Wako, cat. no. 131-14572).
3. Lysis buffer: Laemmli sample buffer with 5 m*M* 2-mercaptoethanol (80 mL Laemmli sample buffer with 10 mL 2-mercaptoethanol).
4. Immobilon-P (Millipore, Billerica, MA; cat. no. IPVH15150).
5. 5 and 20% skim milk.
6. 0.1 *M* PB: dissolve 3.31 g $NaH_2PO_4 \cdot 2H_2O$ and 33.7 g $Na_2HPO_4 \cdot 12H_2O$ in 1 L distilled water.
7. Phosphatase substrate (Amersham, Piscataway, NJ; cat. no. US12387). Rabbit polyclonal antibodies, anti-α-crystallin (1:1000; Stressgen, Victoria, BC Canada; cat. no. SPA-221) and anti-Pax6 (1:200; Santa Cruz, Santa Cruz, CA; cat. no. sc-11357) anti-cellular retinaldehyde-binding protein antibody (1:40,000, kindly provided by John C. Saari, University of Washington, Seattle, WA).
8. Biotinylated antirabbit immunoglobulin (Ig) G conjugated to alkaline phosphatase (Vector Laboratories, Burlingame, CA; cat. no. AP-1000): dilute 1:100 for use.

2.5. Transplantation

1. Papain dissociation system (Worthington Biochemical, Lakewood, NJ; cat. no. LK003150).
2. CM-DiI (chloromethylbenzamido derivatives of 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate; Molecular Probes, Eugene, OR; cat. no. C7001): prepare a 5 g/mL solution in the culture medium.
3. Contact lens (order made by Kyocon, Kyoto, Japan).
4. Head-tracking apparatus (Hayashi Seisakusho, Kyoto, Japan).

3. Methods

3.1. Tissue Culture

3.1.1. Maintenance of STO Cells

1. Prepare a 10-cm dish with confluent STO cells.
2. Remove medium and wash twice with PBS.
3. Add 2 mL 0.05% trypsin-EDTA to the 10-cm dish.
4. Remove trypsin-EDTA immediately.
5. Leave the dish in a culture hood for about 2 min until the cells float with gentle agitation.
6. Add 10 mL DMEM with 10% FBS and pen-strep.
7. Dissociate the cells five times with careful pipetting.
8. Plate 1–2 mL cell suspension to each 10-cm gelatin-coated dish. Add medium to make a 10-mL final volume in each dish. Agitate the dishes gently side to side and back and forth to distribute the cells evenly.
9. Incubate at 37°C until they reach confluency, changing the medium every 2 d.

3.1.2. Preparation of Feeder Layers for ES Cells

3.1.2.1. Day 1

1. Add 50 µL mitomycin C to two 10-cm dishes with confluent STO cells in 10 mL medium.
2. Incubate at 37°C for 1.5–2 h.
3. Remove the medium and rinse with 10 mL PBS.
4. Replace the medium with DMEM with 10% FBS and pen-strep.
5. Incubate at 37°C overnight.

3.1.2.2. Day 2

1. Rinse twice with 10 mL PBS(–).
2. Add 2 mL 0.05% trypsin-EDTA to the 10-cm dishes.
3. Remove trypsin-EDTA immediately.
4. Leave the dishes in a culture hood for approx 2 min until the cells float with gentle agitation.
5. Add 5 mL DMEM with 10% FBS to each of the two plates and collect the cells into a 50-mL conical tube.
6. Centrifuge at 190*g* for 5 min to pellet the cells.
7. Suction off the supernatant with a Pasteur pipet.
8. Resuspend the cells in 10 mL DMEM with 10% FBS and pen-strep by pipetting gently 10 times.
9. Count the number of cells in 50 µL cell suspension.
10. Make a cell suspension of 1.6×10^5 cells/mL and add 4 mL of it to each of the gelatin-coated 6-cm dishes.
11. Gently agitate the dishes side to side and back and forth to distribute the cells evenly.
12. Incubate at 37°C (*see* **Note 5**).

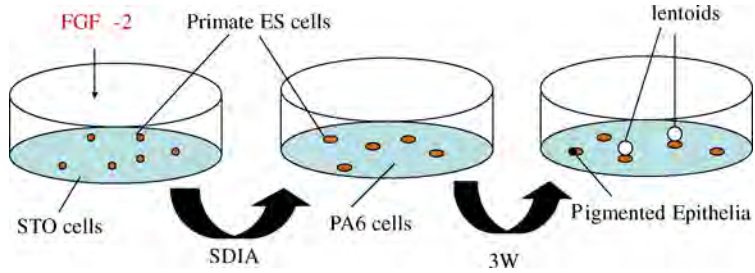

Fig. 1. Stromal cell-derived inducing activity (SDIA). (Please *see* the companion CD for the color version of this figure.)

3.1.3. Maintenance of PA6 Cells

1. Rinse the confluent PA6 cells (in 10-cm noncoated dishes) with PBS (–) once.
2. Add 2 mL 0.05% trypsin 1m*M* EDTA.
3. Remove the trypsin-EDTA immediately.
4. Incubate at 37°C for 5 min.
5. Tap the dish to float the cells.
6. Add 5 mL α-MEM with FBS and gently pipet the cells.
7. Plate 1 mL of cell suspension in a 10-cm noncoated dish and add 7 mL α-MEM with FBS (*see* **Note 6**).

3.1.4. Maintenance of Monkey ES Cells

1. Maintain undifferentiated monkey ES cells on a feeder layer of mitomycin C-treated STO cells in supplemented DMEM/F12.
2. Change the medium every day.

3.1.5. Subculturing of ES Cells

1. Dissociate ES cells with 0.25% trypsin in PBS with 20% KSR and 1 m*M* $CaCl_2$.
2. Transfer onto a new feeder layer.
3. To evaluate the efficacy of bFGF for lentoid formation, add bFGF at concentrations of 2, 4, or 8 ng/mL 3–4 d before plating on PA6 stromal cells.

3.1.6. Induction of ES Cells by the SDIA Method

1. Wash PAb cells twice with differentiation medium.
2. Trypsinize ES colonies for 5 min at 37°C with 0.25% trypsin in PBS.
3. Collect cell pellets by centrifugation at 190*g* for 5 min, resuspend with maintenance medium and incubate 30 min to remove STO cells.
4. Partially dissociated ES cell clumps (10–50 cells/clump) were plated on PA6 cells at a density of 500 clumps per 10-cm dish (**Fig. 1**).
5. Culture in differentiation medium for 3–6 wk, changing the medium every third day (*see* **Note 7**).

3.1.7. Differentiation of ES Cells

After 2–3 wk in differentiation medium, some cells in several colonies of ES cells have pigment, and in the same period amassed cells form transparent protruding bodies of varying size. These cells were shown to be RPE cells (**Fig. 2**) ***(4)*** (*see* **Note 8**) and lentoid bodies (**Fig. 3**) ***(5)***.

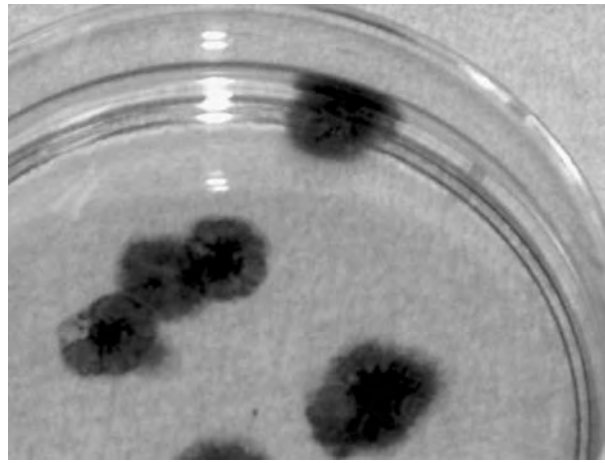

Fig. 2. Monkey embryonic stem cell-derived retinal pigment epithelial cells.

Fig. 3. Monkey embryonic stem cell-derived lentoids.

3.1.7.1. Pigment Cell Formation, Isolation, and Expansion

1. Coat dishes with synthetic matrix (Matrigel) or collagen.
2. Selectively remove the pigmented cells with a disposable scalpel.
3. Plate on the coated dishes in DMEM supplemented with 10% FBS and 20 ng/mL bFGF.

3.1.7.2. Effects of Exogenous FGF-2 on Lentoid Induction

To evaluate the efficacy of bFGF for lentoid formation, bFGF was added at concentrations of 2, 4, or 8 ng/mL 3–4 d before plating on PA6 stromal cells. Although at 20 d of induction, there were no significant differences between the different concentrations,

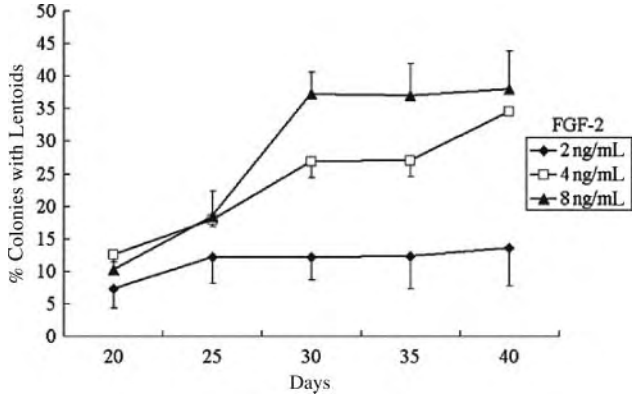

Fig. 4. Effects of exogenous fibroblast growth factor-2 on lentoid induction.

after 30 d the percentage of colonies containing lentoids increased in a dose-dependent manner with increasing bFGF concentrations in maintenance undifferentiated ES cell cultures. At 40 d of induction, the proportion of colonies containing lentoids (% colonies with lentoids) were 14 ± 6% (n = 911) in 2 ng/mL FGF-2, 35 ± 2% (n = 980) in 4 ng/mL, and 38 ± 7% (n = 960) in 8 ng/mL (**Fig. 4**).

3.1.7.3. Effects of the Colony Density on Lentoid Induction

The percentage of colonies containing lentoids increased in proportion to the density of ES colonies added at the start of the culture period (**Fig. 5**). After 30 d of induction, the numbers of lentoids induced by ES cell differentiation in cultures plated at a high density on PA6 cells (200–300 colonies/10-cm dish) were greater than those at low density (70–150 colonies/10-cm dish) at each concentration of bFGF in maintenance undifferentiated ES cultures. The induction of pigmented epithelial cells was also increased in high colony density cultures.

3.2. Analysis of Differentiation

3.2.1. In Vitro Immunofluorescence

1. Set up and maintain cells in chambered slides.
2. Fix the cells in 4% paraformaldehyde for 1 h at 4°C.
3. Rinse in 0.1 *M* phosphate buffer for 10 min.
4. Incubate chambered slides in a humidified chamber at room temperature with 20% skim milk in 0.1 *M* PB-saponin for 1 h to block nonspecific antibody binding.
5. Incubated for 24 h at 4°C with primary antibody (*see* **Note 9**) diluted in 5% skim milk in 0.1 *M* PB-saponin.
6. Rinse three times in 0.1 *M* PB.
7. Incubate with the secondary antibody diluted in 0.1 *M* PB-saponin with 5% skim milk for 1 h at room temperature.
8. Rinse three times with 0.1 *M* PB.
9. Remove the chambers from slides and mount with glycerol-PBS (1:1).
10. Observe by laser scanning confocal microscope.

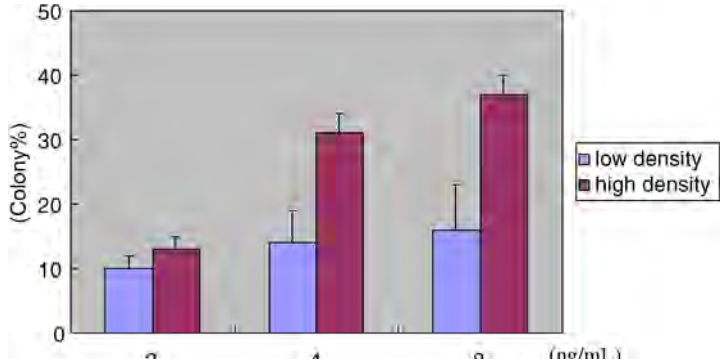

Fig. 5. Effects of the colony density on lentoid induction. (Please *see* the companion CD for the color version of this figure.)

3.2.2. Reverse Transcriptase Polymerase Chain Reaction

3.2.2.1. Trizol RNA Extraction

1. Lyse cells in the tissue culture dish by adding 200 µL Trizol.
2. Remove the cells with a cell scraper, then pipet the solution several times to collect all the cells. Transfer the volume to a sterile culture tube. Incubate the samples for 5 min at room temperature.
3. Add 0.2 mL chloroform (ribonuclease [RNase] free) per 1 mL Trizol used. Shake the tubes for 15 s, then incubate at room temperature for 3 min.
4. Centrifuge at 12,000*g* for 15 min at 4°C. The mixture will separate into a lower phenol:chloroform phase, an interface, and a colorless upper aqueous phase.
5. Transfer the aqueous phase into a fresh tube. Precipitate the RNA by adding 0.5 mL RNase-free isopropanol per 1 mL Trizol originally added. Incubate the samples for 10 min at room temperature.
6. Centrifuge at 12,000*g* for 10 min at 4°C. The RNA forms a pellet on the bottom and the side of the tube.
7. Remove the supernatant and wash the pellet with RNase-free 75% ethanol. Use 1 mL ethanol for each 1 mL Trizol used originally. Vortex to mix the sample and centrifuge at 7500*g* for 5 min at 4°C.
8. Air-dry the pellet and resuspend in DEPC-H_2O (*see* **Notes 10** and **11**).

3.2.2.2. Denature RNA

1. Mix 1 µL RNA with 2 µL DEPC-H_2O in a 100-µL PCR tube.
2. Vortex and pulse.
3. Place tubes in a thermal cycler and run the RNA at 65°C for 10 min, then chill on ice.

3.2.2.3. Reverse Transcription

1. Gently pipet the bulk first-strand complementary DNA reaction mix to obtain a uniform suspension and add 5 µL to a sterile 0.5-mL microcentrifuge tube.
2. Add 1 µL dithiothreitol solution, 1 µL random hexamer, and 8 µL heat-denatured RNA. Pipet up and down several times to mix.
3. Incubate at 37°C for 1 h.

3.2.2.4. Polymerase Chain Reaction

1. Prepare the PCR mixture (50 µL final volume): 0.5 µL template DNA (approx 10 ng), 5.0 µL 2.5 m*M* dNTPs, 1.0 µL 10X polyelectrolyte complex buffer, 1.0 µL 3′ primer (25 pmol/µL) (*see* **Note 12**), 1.0 µL 5′ primer (25 pmol/µL) (*see* **Note 10**), 0.5 µL *Taq* DNA polymerase, and 37 µL distilled water.
2. Run samples: 94°C for 2 min; three times at 94°C for 30 s, 58°C for 30 s, 72°C for 30 s; 72°C for 7 min; and 4°C for 5 min and hold at 15°C.

3.2.3. Western Blot Analysis

3.2.3.1. Making Cell Lysate

1. Harvest cells by scraping.
2. Lyse cells in 500 µL of lysis buffer.
3. Homogenize cell suspensions on ice for 5 min. Cell homogenates can be stored at 80°C.

3.2.3.2. Western Blot Analysis

1. Separate proteins on sodium dodecyl sulfate polyacrylamide gels and transfer to Immobilon-P membranes by electrophoresis.
2. Block nonspecific antibody binding by incubation with 20% skim milk in 0.1 *M* PB for 1 h.
3. Incubate blots at room temperature with primary antibody diluted in 5% skim milk in 0.1 *M* PB for 1 h.
4. Detect primary antibody binding with a biotinylated antirabbit IgG conjugated to alkaline phosphatase (1:100) by the avidin-biotin complex method.
5. Rinse three times in 0.1 *M* PB.
6. Develop the blots with phosphatase substrate according to the manufacturer's protocol.

3.3. Analysis of Cell Function In Vivo (RPE)

3.3.1. Transplantation Procedures

1. Collect patches of ESPEs by carefully cutting the peripheral margins with disposable scalpels.
2. Gently dissociate the patches of ESPEs with the Papain dissociation system according to the manufacturer's protocol.
3. Incubate dissociated ESPEs in CM-DiI solution for 20 min at 37°C.
4. Wash labeled ESPEs three times with PBS.
5. Centrifuge the cells and concentrate to approx 10,000 cells/L in PBS (*see* **Note 13**).
6. Inject ESPE cells suspended in 3 µL PBS transsclerally into the dorsotemporal subretinal space of anesthetized 4-wk-old RCS rats (*see* **Notes 14–17**).

3.3.2. In Vivo Immunofluorescence

1. Harvest eyes ($n = 4$ for each group) 8 wk after transplantation (at age 12 wk) and fix in 4% paraformaldehyde for 2 h at 4°C.
2. Cut 16-µm sections with a cryostat. Stain and process for light microscopy (*see* **Note 12**).
3. Measure the maximum thickness of the outer nuclear layer in the dorsotemporal and ventronasal retina ($n = 4$ animals for each group) and analyze the differences.

3.3.3. Visual Function Test

Animals ($n = 4$ animals for each group) were tested at 8 wk after transplantation at 12 wk of age before they were killed. For behavioral assessment, a head-tracking

Fig. 6. Visual function test of rats (a head-tracking apparatus).

apparatus (**Fig. 6**) that consisted of a circular drum rotating around a stationary holding chamber containing the animal was used. The test was done by a person who did not know which animals had received RPE transplantation or sham operation.

1. Set the speed of rotation of the drum with vertical black-and-white stripes (10° each) at 2, 4, and 8 rpm.
2. Mount a video camera above the apparatus to record the head movements. The total amount of head-tracking time is determined at speeds of 2, 4, and 8 rpm during a 4-min test period for each speed.
3. Analyze behavioral data with the Mann-Whitney test.

4. Notes

1. Prepare mitomycin C in the tissue culture hood. Add 5 mL PBS to a 10-mg vial, recap, and shake. Sterilize it through 0.2-µm pore filter attached to a syringe. Prepare 110-µL aliquots and store at –20°C. For use, thaw the aliquots in foil to protect from light.
2. Use a 1:1 mixture of DMEM and Ham's nutrient mixture F-12.
3. Sterilized gelatin solution is plated in culture dishes (7 mL for a 10-cm dish, 3 mL for a 6-cm dish). Incubate for more than 1 h. Remove the gelatin solution before use.
4. A humidified chamber can be made by placing a piece of wet paper towel in a plastic box with a glass slide placed on top of the paper.
5. Use within 4 d after plating; discard after 4 d.
6. Plate on gelatin-coated dishes before using as a feeder cell layer.
7. Unlike mouse ES cells, monkey ES cells do not form colonies from single cells on PA6 cells *(6)*. Low cloning efficiency has been also reported for human ES cells. It is therefore necessary to plate clumps of 10–50 undifferentiated ES cells for starting induction.
8. With the SDIA method, pigmented cells emerge after 2–3 wk, reproducibly exhibiting a hexagonal shape, with each cell containing a significant amount of melanin pigments.
9. The reactivity of the antibodies was confirmed by using rat lens or RPE as a positive control. The pigmented cells showed ZO-1 immunoreactivity in hexagonal shapes (**Fig. 7**) The lentoid cells showed α-crystallin and Pax6 immunoreactivity.

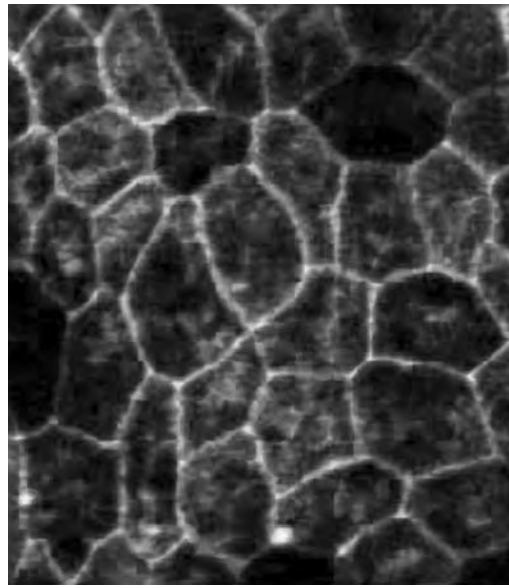

Fig. 7. ZO-1 immunoreactivity in the pigment epithelial cells derived from monkey embryonic stem cells.

10. Find the OD at A_{260} and A_{280}. The A_{260}:A_{280} should be between 1.6 and 2.
11. Determine the concentration of the sample using the following equation: [] μg/μL = [A_{260} × 40 × Dilution factor]/1000.
12. Primer sequences: for RPE65, 5-TGG AGT CTT TGG GGA GCC AA-3 and 5-CTC ACC ACC ACA CTC AGA AC-3; for cellular retinaldehyde-binding protein (CRALBP), 5-GTG GAC ATG CTC CAG GAT TC-3 and 5-CCA AAG AGC TGC TCA GCA AC-3; for Mertk, 5-GGG AGA TCG AGG AGT TTC TC-3 and 5-CGG CCT TGG CGG TAA TAA TC-3; for actin, 5-CTT CAA CAC CCC AGC CAT GT-3 and 5-ACT CCT GCT TGC TGA TCC AC-3.
13. The viability of the ESPEs after these procedures was more than 95%, as assessed by trypan blue exclusion.
14. All transplantations are made into the left eye. Sham-treated RCS rats received the same amount of carrier medium.
15. Confirm transplantation into the subretinal space by direct observation of the rat fundus with a contact lens and select those with successful transplantation for histologic analyses and behavioral tests.
16. All the animals were maintained on oral cyclosporine (200 mg/L in drinking water; Calbiochem, Darmstadt, Germany) from 2 d before transplantation until they were sacrificed.
17. The blood cyclosporine levels in these animals were measured by SRL Inc. (Tokyo, Japan).

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29

Differentiation of Rhesus Monkey Embryonic Stem Cells in Three-Dimensional Collagen Matrix

Silvia Sihui Chen, Roberto P. Revoltella, Joshua Zimmerberg, and Leonid Margolis

Summary

During normal embryogenesis, embryonic stem cells (ESCs) reside in the context of complex three-dimensional tissue structures, in particular of extracellular matrices (ECMs), which determine cell migration, proliferation, and differentiation. Therefore, to study ESC differentiation in an in vivo-like microenvironment, three-dimensional culture systems are necessary. Here, we developed protocols for ESC cultures in three-dimensional systems consisting of collagen matrices (collagen gels and porous collagen sponges) to investigate the mechanisms of ESC differentiation as well as the formation of tissue-like structures. In collagen matrices, ESCs differentiate into neural, epithelial, and endothelial lineages. In this system, ESCs form various tissue-like structures. The abilities of ESCs to form such structures in two chemically similar but topologically different matrices are different. In particular, in collagen gels ESCs form gland-like circular structures, whereas in collagen sponges ESCs are scattered through the matrix and form aggregates. To mimic the in vivo situation further, we developed a protocol for co-cultures of ESCs with human dermal fibroblasts or keratinocytes in collagen matrixes. Co-culture with fibroblasts in collagen gel facilitates ESC differentiation into cells of a neural lineage expressing nestin, neural cell adhesion molecule (NCAM), and class III β-tubulin. In collagen sponges, keratinocytes facilitated ESC differentiation into cells of an endothelial lineage expressing factor VIII. Thus, the developed protocols promote ESC differentiation into a particular lineage, accompanied by the formation of tissue-like structures. Three-dimensional culture systems are a valuable tool for directing ESC differentiation and the formation of organs and tissues.

Key Words: Collagen matrix; embryonic stem cell; tissue-like structure.

1. Introduction

The future use of human embryonic stem cells (ESCs) for therapy requires a deep understanding of the basic mechanisms of cell differentiation and tissue formation as well as the development of methods to control ESC differentiation into particular lineages. Nonhuman primate ESCs have a notable advantage over human ones; although the

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physiology of both types of cells is similar, experiments with the former are subject to fewer ethical restrictions. Therefore, rhesus monkey ESCs become an important model for the study of human ESCs.

Most of the in vitro studies of both human and rhesus monkey ESC differentiation have been performed in monolayer cultures; in vivo, this process occurs in the context of three-dimensional tissues. Three-dimensional systems provide an environment for ESCs to form tissues and organs. For example, under experimental conditions in severe combined immunodeficient mice, rhesus monkey ESCs differentiated and formed teratomas with structures of ectodermal origin resembling neural tubes, embryonic ganglia, and brain-like gray matter *(1)*, as well as structures of endoderm-derived tissues, including intestinal and ductal epithelium and pancreas *(2)*. Apparently, ESCs migrate within the tissues and interact with the tissues' resident cells as well as with noncellular elements, particularly extracellular matrices (ECMs), which are vastly different from monolayer cultures. ECMs such as collagens play important roles in cell differentiation by providing biological signals to promote and maintain cell differentiation *(3,4)*. Together with growth factors, ECMs may create distinct cellular microenvironments, or "niches," that locally regulate ESC proliferation and differentiation.

To investigate differentiation of ESCs as well as the formation of tissue-like structures in the context of in vivo-like three-dimensional systems, we developed protocols for ESC differentiation in three-dimensional collagen matrices. Briefly, we seeded rhesus monkey ESCs on type I collagen in two structurally different forms: a gel and a sponge. Under these conditions, ESCs migrate into the collagen matrices, proliferate, differentiate, and form various tissue-like three-dimensional structures. To mimic the in vivo situation further, we co-cultured ESCs in collagen matrices with human dermal fibroblasts or keratinocytes. This protocol facilitates the differentiation of ESCs into a particular lineage, accompanied by the formation of tissue-like structures. Three-dimensional culture systems are a valuable tool for directing ESC differentiation and the formation of organs and tissues for transplantation.

2. Materials

2.1. Maintenance of Undifferentiated Rhesus Monkey ESCs

1. Rhesus monkey ESCs R366.4 (WiCell, Madison, WI; http://www.wicell.org/).
2. ESC culture medium (1X; store at 4°C): for a final volume of 1 L, 800 mL of knockout Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Rockville, MD; cat. no. 10829-018) supplemented with 200 mL defined fetal bovine serum (FBS) (Hyclone, Logan, UT; cat. no. SH30070.03), 10 mL nonessential amino acids (Invitrogen, cat. no. 11140-035), 10 mL 200 m*M* L-glutamine (Invitrogen, cat. no. 25030-081), and 2 mL 55 m*M* β-mercaptoethanol (Invitrogen, cat. no. 31350-010).
3. Mouse embryonic fibroblasts (MEFs) (Cell Essential Inc., Boston, MA; http://www.cellessentials.com/).
4. MEF culture medium (1X; store at 4°C): for a final volume of 1 L, 900 mL DMEM (Invitrogen, cat. no. 11965-092) supplemented with 100 mL FBS (Gemini Bioproducts, Woodland, CA; cat. no. 100-106) and 10 mL penicillin and streptomycin (Invitrogen, cat. no. 15070-063).
5. Gelatin (0.1%) in ultra pure water (Specialty Media, Phillipsburg, NJ; cat. no. ES-006-B).

6. Trypsin and ethylenediaminetetraacetic acid (EDTA) (0.05% trypsin, 0.53 m*M* EDTA · 4Na) (Invitrogen, cat. no. 25300-112).
7. Mitomycin C (8 µg/mL in DMEM) (Sigma, St. Louis, MO; cat. no. M-4287).
8. Collagenase IV (Invitrogen, cat. no. 17104-019): 1 mg/mL in knockout DMEM for splitting ESCs.
9. Phosphate-buffered saline (PBS) (calcium and magnesium free) (Invitrogen, cat. no. 14190-144).
10. Six-well plates (Nalge Nunc Inc., Naperville, IL; cat. no. 152795).
11. T-80 flasks (Nalge Nunc, cat. no. 156499).

2.2. Control Differentiation of ESCs

1. High-grade malignant human keratinocyte HaCaT-ras clone A5RT.1 cells (a gift from Drs. Petra Boukamp and Norber Fusenig from the German Cancer Institute, Heidelberg, DE).
2. Human dermal fibroblasts HPI.l (isolated by Dr. Roberto P. Revoltella).
3. Base medium: same as MEF culture medium (*see* **Subheading 2.1.**, **step 4**).
4. Sodium L-ascorbate (Sigma, cat. no. A-4034).
5. Recombinant human (rh) granulocyte macrophage colony-stimulating factor (GM-CSF) (a gift from Dr. F. Bertolero of the former Farmitalia-Carlo Erba, now Upjohn, Milan, Italy).
6. Rat tail type I collagen (Boehringer-Mannheim/Roche Diagnostics, Indianapolis, IN; cat. no. 1179 179).
7. Sterile filtered 1 *N* NaOH.
8. Sterile filtered 0.2% acetic acid.
9. Sterile Gelfoam sponges (Pharmacia and Upjohn, Kalamazoo, MI; cat. no. NDC0009-0315-03) from purified pork skin gelatin.
10. Six-well inserts (Corning Costar, Corning, NY; cat. no 3450).
11. Two-well chamber slides (Nalge Nunc, cat. no.177429).

2.3. Immunohistochemical Analysis

1. Citri-solv (Fisher, Fairlawn, NJ; cat. no. 22-143975).
2. Target retrieval buffer (Dako, Carpinteria, CA; cat. no. S1699).
3. Primary antibodies and their dilutions (*see* **Table 1**).
4. Vector MOM mouse immunoglobulin G (IgG) blocking reagent (Vector Laboratories, Burlingame, CA; cat. no. BMK-2202).
5. 1X Tris-buffered saline (TBS) (Biofluids, Camarillo CA; cat. no. 616NS-000) supplemented with 1% bovine serum albumin (BSA) (Sigma, cat. no. A-0336).
6. The LSAB+ immunoperoxidase system (Dako, cat. no. K0779) in which biotinylated anti-rabbit, mouse, and goat immunoglobulin is used as a secondary antibody, streptavidin conjugated to horseradish peroxidase is used as a link agent, and 3,3′-diaminobenzidine is used as a chromogen.
7. Mayer hematoxylin (Sigma, cat. no. 51275).
8. Powerblock (Biogenex, San Ramon, CA; cat. no. HK085-5K).
9. Xylene-based mounting medium (Richard-Allan Scientific, Kalamazoo, MI; cat. no. 8312-4).
10. Water-based mounting medium (Dako, cat. no. 002972).

3. Methods

3.1. Preparation of MEF Feeder Layer

1. Culture MEFs with 12 mL MEF medium in T-80 flasks until confluent.
2. Add 2 mL 0.1% gelatin in ultra pure water into each well of a six-well plate and keep overnight.

Table 1
Primary Antibodies for the Protocol

Antibody	Specificity	Species	Clonality	Dilution	Source
Anti-SSEA-3 (MC-631)	Undifferentiated ESCs	Rat	Monoclonal	1:5	Developmental Hybridoma Studies Bank (DHSB), University of Iowa, Iowa City, IA; cat. no. MC-631
Anti-SSEA-4 (MC-813-70)	Undifferentiated ESCs	Mouse	Monoclonal	1:10	DHSB, cat. no. MC-813-70
Anti-class III β-tubulin (TU-20)	Neurons at early development stage	Mouse	Monoclonal	1:100	Chemicon, Temecula, CA; cat. no. MAB1637
Anti-NCAM	Neural/developing skeletal muscle cells	Rabbit	Polyclonal	1:300	Chemicon, cat. no. AB5032
Anti-nestin	Neuroepithelial/ neural stem cells	Rabbit	Polyclonal	1:400	Chemicon, cat. no. AB5922
Anti-chromo-granin A	Neuronal/ neuroectodermal/ neural-endocrine cells	Rabbit	Polyclonal	1:150	Dako, Carpinteria, CA; cat. no. A0430
Anti-factor VIII	Endothelial cell	Rabbit	Polyclonal	1:200	Dako, cat. no. A0082
Anti-vimentin (V9)	Mesenchymal/ endothelial/ glial/neural stem cells	Rabbit	Monoclonal	1:50	Vector Laboratories, Burlingame, CA; cat. no. NCL-VIM-V9
Anti-cytokeratins (AE1/AE3)	Epithelial cells	Mouse	Monoclonal	1:50	Dako, cat. no. M3515
Anti-Ki-67 (MIB-1)	Proliferating cells	Mouse	Monoclonal	1:50	Dako, cat. no. M0722

3. Treat MEFs with 8 µg/mL mitomycin C for 2 h (*see* **Note 1**). Wash with 1X PBS four times to remove mitomycin C.
4. Add 2 mL trypsin-EDTA into a T-80 flask and incubate at 37°C for 5 min.
5. Add 5 mL MEF medium and collect mitomycin C-treated MEFs. Centrifuge at 400*g* for 5 min.
6. Discard supernatant, add 5 mL culture medium to rinse cells, and spin again.
7. Discard supernatant and resuspend MEFs at 150,000 cells/mL in ESC medium.
8. Add 2 mL MEF suspension into each well of the gelatin-treated six-well plate to reach 300,000 cells/well and culture overnight.

3.2. Culture of Undifferentiated ESCs

Undifferentiated rhesus monkey ESCs R366.4 are routinely maintained as previously described (*5*). Briefly, ESCs are growing as colonies on top of an MEF feeder layer. To passage undifferentiated ESCs cultured in six-well plates:

1. Remove ESC culture medium from culture wells.
2. Add 1 mL 1 mg/mL collagenase IV in knockout DMEM into each well of the six-well plate and incubate at 37°C for 10 min.
3. Add 1 mL ESC culture medium to each well and gently scrape cells with a 5-mL pipet. Transfer cells to a 50-mL tube.
4. Centrifuge cells for 2 min at room temperature at 200*g*.
5. Discard supernatant, add 5 mL culture medium to rinse cells, and spin again.
6. Discard supernatant and resuspend cells in an appropriate volume of ESC medium (*see* **Note 2**). Mix the cell suspension by pipetting to break large ESC colonies into small ones (*see* **Note 3**).
7. Transfer 2 mL ESC suspension to each well of the MEF-seeded six-well plates.
8. Rock each plate side to side gently to evenly disperse ESC colonies just prior to placing it in the incubator.
9. Change medium every day.

3.3. Control of ESC Differentiation

3.3.1. Culture of ESCs on Two-Dimensional Chamber Slides

ESCs cultured as monolayers on two-well chamber slides are needed to compare two- and three-dimensional cultures and to verify their pluripotency.

1. Release the ESCs from the six-well plate with collagenase IV (1 mg/mL) as described in **Subheading 3.2., steps 1–5**.
2. Suspend the ESCs in three different growth media (*see* **Note 4**) consisting of one of the following:
   a. Base medium.
   b. A5RT.1-conditioned medium obtained as follows: culture A5RT.1 cells as a monolayer with 12 mL base medium in a T-80 flask. Collect the 2-d conditioned medium, centrifuge, filter (0.22-µm pore size filter), dilute 1:3 with fresh base medium.
   c. HPI.1-conditioned medium obtained as follows: culture HPI.l cells as a monolayer with 12 mL base medium in a T-80 flask. Collect the 2-d conditioned medium, centrifuge, filter (0.22-µm pore size filter), and dilute 1:3 with fresh base medium.
3. Transfer 1 mL ESC suspension to each well of the two-well chamber slides. Change the medium every 3 d.
4. Remove culture medium from chamber slides on d 8. Rinse chamber slides with PBS, fix in 4% formaldehyde for 30 min or in a 1:1 mixture of cold methanol and acetone for 10 min as recommended by the antibody manufacturers, wash in PBS again, and keep in PBS at 4°C until immunohistochemical analysis.

3.3.2. Culture of ESCs on Three-Dimensional Collagen Gel

3.3.2.1. Culture of HPI.1 Cells as a Feeder Layer for Co-Culturing With ESC

1. Culture HPI.1 cells with 12 mL base medium in a T-80 flask until confluent. Change the medium every 3 d.
2. Remove culture medium and rinse HPI.1 cells once with PBS.
3. Add 2 mL trypsin-EDTA into the T-80 flask and incubate at 37°C for 5 min.
4. Add 5 mL base medium and collect HPI.1 cells. Centrifuge at 400*g* for 5 min.
5. Discard supernatant, add 5 mL culture medium to rinse cells, and spin again.
6. Discard supernatant and resuspend HPI.1 at 150,000 cells/mL in base medium.
7. Add 2 mL HPI.1 cell suspension into each well of the six-well plate and culture for 24 h before ESC seeding.

3.3.2.2. Preparation of Collagen Gel for ESC Culture

1. Add 3.3 mL sterile 0.2% acetic acid to 10 mg dry rat tail collagen to reconstruct the 3 mg/mL collagen solution. Store at 2–8°C at least 24 h before use. Avoid pipetting and mixing.
2. Prepare a container filled with ice. Perform all the following procedures on ice (*see* **Note 5**).
3. Add the components in a 50-mL conical tube in the following order to obtain 5 mL of a 2.4 mg/mL (final concentration) collagen solution: 0.5 mL DMEM and 0.5 mL FBS; mix well. Add 4 mL 3 mg/mL collagen solution (should be added slowly) and sterile 1 *N* NaOH (should be added drop by drop until the color of the mixture changes from yellow to orange/red).
4. Add 1 mL 2.4 mg/mL collagen solution into the six-well culture insert.
5. Add 2 mL base medium supplemented with 50 µg/mL ascorbate into the outside compartment of the culture insert only. Incubate at 37°C overnight.

3.3.2.3. Embedding of HPI.1 Cells in Collagen Gel for Co-Culturing With ESCs

1. Release HPI.1 from the T-80 flask as in **Subheading 3.3.2.1.**, **steps 2–5**. Suspend the HPI.1 cells at 1×10^6/ mL in DMEM.
2. Follow the same procedure as in **Subheading 3.3.2.2.**, **steps 1–4**, except substitute HPI.1 cell suspension for DMEM to obtain 5 mL 2.4 mg/mL (final concentration) collagen solution with 0.1×10^6 HPI.1 cells/mL embedded.
3. Add 2 mL base medium supplemented with 50 µg/mL ascorbate in the outside compartment of the culture insert only. Keep the collagen gel embedded with HPI.1 at 37°C in a 5% CO_2 air atmosphere overnight.

3.3.2.4. Culture of ESC With or Without HPI.1 as a Feeder Layer

1. Release ESCs from the six-well plate with collagenase IV (1 mg/mL) as in **Subheading 3.2.**, **steps 1–5**, after the collagen solution has solidified.
2. Suspend one well of ESCs cultured in the six-well plate with 2 mL base medium.
3. Rinse the solidified collagen gel in the culture insert with base medium once and remove the medium from the outside compartment of the insert.
4. Add 1 mL ESC suspension on top of the collagen gel in each of the six-well culture inserts.
5. Transfer the insert into the six-well plate with or without a 24-h-old HPI.1 feeder layer at the bottom of the well. Add 2 mL base medium supplemented with 50 µg/mL ascorbate in the outside compartment of the insert.
6. Remove all the medium from the inside compartment of the insert after the ESCs settle down (usually overnight is enough). Rinse the inside compartment with fresh base medium once and remove all medium from both the inside and the outside compartments. Add base medium supplemented with 50 µg/mL ascorbate in the outside compartment of the insert only.
7. Culture ESCs at the air-fluid interface (**Fig. 1**). Change medium every 2 d.
8. Remove all culture medium after 7 and 19 d of culture. Rinse the inserts with PBS, fix in 4% formaldehyde in PBS at 4°C for 3 d, wash with PBS, and keep in PBS at 4°C until paraffin embedding and sectioning.

3.3.2.5. Culture of ESC With HPI.1 Embedded in Collagen Gel

1. Release and resuspend ESCs as previously described (*see* **Subheading 3.2.**, **steps 1–5**).
2. Rinse the collagen gel embedded with HPI.1 cells in the culture insert with base medium once and remove the medium from the outside compartment of the insert.

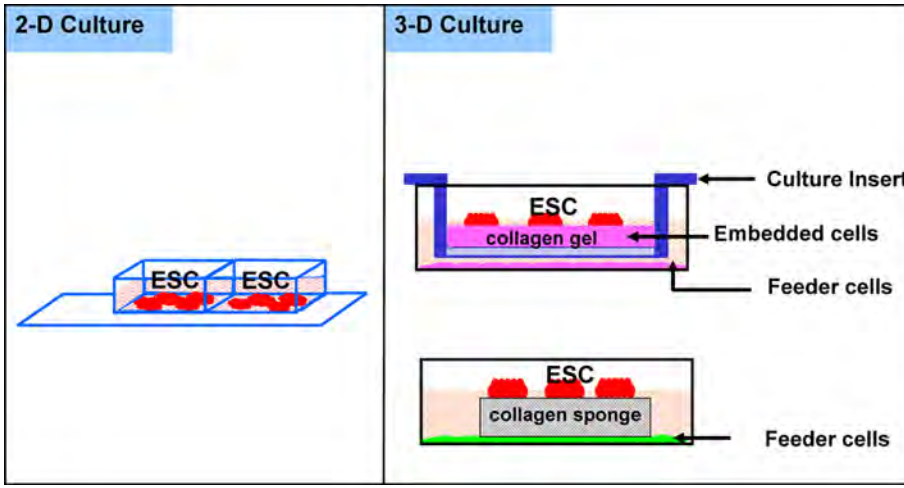

Fig. 1. Experimental procedure. Undifferentiated embryonic stem cells (ESCs) cultured on mitomycin C-treated mouse embryonic fibroblast were transferred onto two-dimensional chamber slides or onto one of two types of three-dimensional collagen I matrixes: collagen gel or collagen sponge. In some experiments, ESCs growing on collagen matrixes were co-cultured with human dermal fibroblasts or keratinocytes in the presence or absence of granulocyte macrophage colony-stimulating factor. (Please *see* the companion CD for the color version of this figure.)

3. Add 1 mL ESC suspension on top of the collagen gel embedded with HPI.1 cells in each of the six-well culture inserts.
4. Add 2 mL base medium supplemented with 50 µg/mL ascorbate in the outside compartment of the insert.
5. Remove all the medium from the inside compartment of the insert after the ESCs settle (usually overnight is enough). Rinse the inside compartment with fresh base medium once and remove all medium from both the inside and the outside compartments. Add base medium supplemented with 50 µg/mL ascorbate in the outside compartment of the insert only.
6. Culture ESCs at the air-fluid interface (**Fig. 1**). Change medium every 2 d.
7. Remove all culture medium after 7 and 19 d of culture. Rinse the inserts with PBS, fix in 4% formaldehyde in PBS at 4°C for 3 d, wash with PBS, and keep in PBS at 4°C until paraffin embedding and sectioning.

3.3.3. Culture of ESCs on Three-Dimensional Collagen Sponge

3.3.3.1. Culture of A5RT.1 Cells as a Feeder Layer for Co-Culturing With ESCs

1. Culture A5RT.1 cells with 12 mL base medium in a T-80 flask.
2. Remove culture medium and rinse A5RT.1 cells once with PBS.
3. Add 2 mL trypsin-EDTA into the T-80 flasks and incubate at 37°C for 5 min.
4. Add 5 mL base medium and collect A5RT.1 cells. Centrifuge at 400*g* for 5 min.
5. Discard the supernatant, add 5 mL culture medium to rinse the cells, and spin again.
6. Discard the supernatant and resuspend the A5RT.1 cells at 150,000 cells/mL in base medium.

7. Add 2 mL A5RT.1 cell suspension into each well of the six-well plate and culture for 24 h before ESC seeding.

3.3.3.2. Culture of ESCs With or Without HPI.1 or A5RT.1 Cells as a Feeder Layer

1. Moisturize sterile Gelfoam sponges with base medium just before use by soaking and pushing out air bubbles trapped in the sponge with forceps (*see* **Note 6**). Cut each sponge into four 20 × 15 × 7 mm pieces and transfer each piece to a well of a six-well plate with or without an HPI.1 or A5RT.1 feeder layer.
2. Release ESCs as previously described (*see* **Subheading 3.2.**, **steps 1–5**). Suspend one well of ESC cultured in the six-well plate with 250 µL base medium.
3. Seed ESCs in six distinct spots on top of each piece of collagen sponge (apply 20 µL ESC suspension at each spot) (**Fig. 1**).
4. Carefully add 3 mL base medium supplemented with 50 µg/mL ascorbate into each well to cover the sponge (**Fig. 1**). For culture with rhGM-CSF, exogenous 20 ng/mL rhGM-CSF is added to each of the cultures.
5. Culture at 37°C in a 5% CO_2 atmosphere for 24 d. Change medium every 2 d.
6. Remove culture medium on day 24. Wash with 1X PBS and fix the collagen sponges in 4% formaldehyde in PBS at 4°C for 3 d. Wash with PBS again and keep the fixed sponges in PBS at 4°C until paraffin embedding and sectioning.

3.4. Immunohistochemical Analysis

3.4.1. Culture of ESCs in Two-Dimensional Chamber Slides

To characterize the cell types, immunohistochemistry is necessary. We routinely stain cells for nestin, NCAM, class III β-tubulin, and cytokeratin (AE1/AE3). These markers are characteristic of neural precursor cells ***(6)***, neural cells ***(7)***, immature neurons just after the last mitosis ***(8,9)***, and epithelial cells ***(10)***, respectively. Also, we stain cells with anti-Ki-67 (MIB-1) antibodies, which recognize a nuclear antigen expressed in dividing cells ***(11)***. Other differentiation markers can be studied under a similar staining protocol:

1. Aspirate PBS and rinse the fixed cells twice with TBS.
2. Permeabilize the cells with 0.1% Triton X-100 in 1X PBS for 10 min for intracellular staining.
3. Dilute antibodies in TBS plus 1% BSA.
4. Block in Powerblock supplement with 5% FBS or 1–5% goat serum and 0.5% BSA for 10–60 min as recommended by the antibody manufacturers.
5. Follow the staining instructions in the DAKO LSAB+ kit.
6. Counterstain with Mayer hematoxylin for 2 min. Wash in water and dip in PBS 10–12 times.
7. Wash in water.
8. Mount with water-based mounting medium (*see* **Note 7**).

Under the protocol described in this section, on two-dimensional chamber slides, ESCs differentiate, migrate out from original colonies, divide, and form clusters of adherent cells of distinct morphologies. Epithelial-like cells do not express nestin, NCAM, and class III β-tubulin, but they are positive for cytokeratin (**Fig. 2A**). Cells at the periphery of ESC colonies, which appear to be morphologically differentiated, express nestin (**Fig. 2B**). Cells that appear to be of neuronal morphology strongly express NCAM (**Fig. 2C**) and class III β-tubulin (**Fig. 2D**). Immunostaining confirms the morphological evidence of

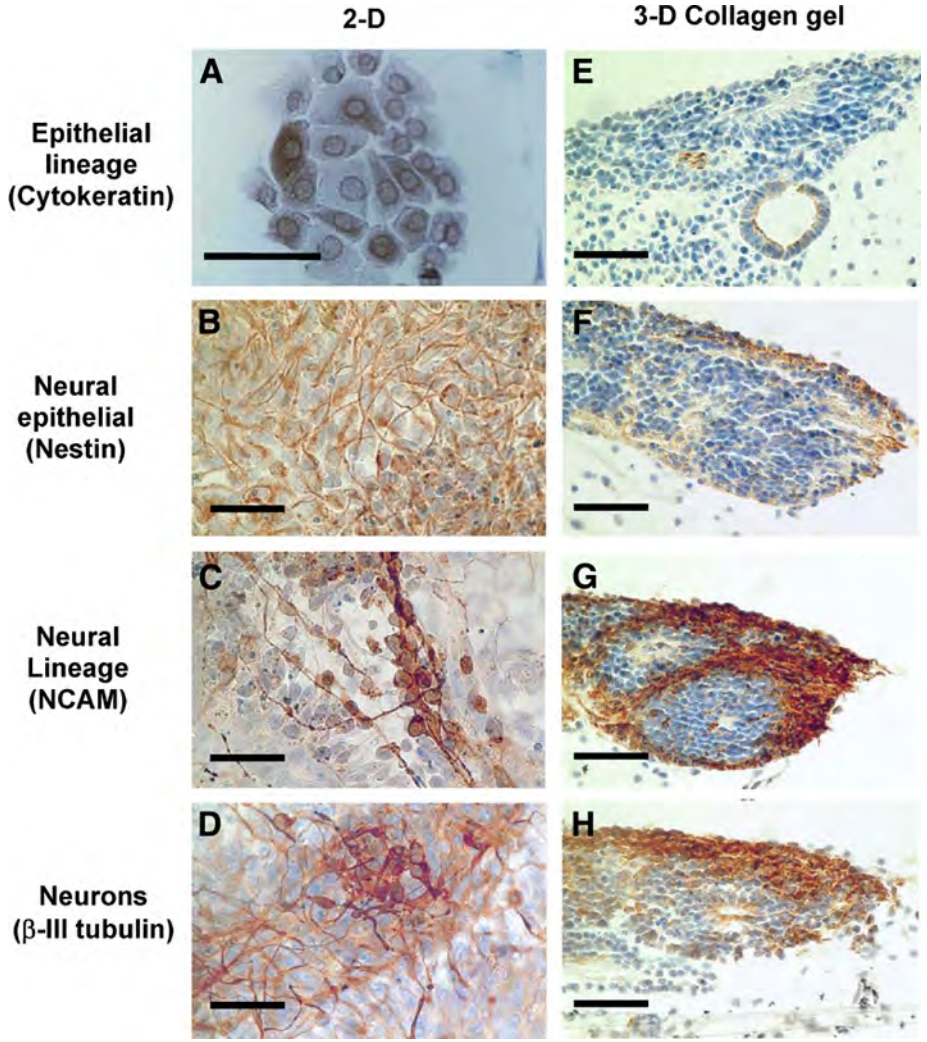

Fig. 2. Immunohistochemical staining of embryonic stem cells cultured on two-dimensional chamber slides for 8 d or on type I collagen gel for 19 d. Cells are stained for cytokeratin (**A**,**E**), nestin (**B**,**F**), NCAM (**C**,**G**), and class III β-tubulin (**D**,**H**). Scale bars = 50 µm except in panel **A** (25 µm) (*see* **Subheadings 3.4.1.** and **3.4.2.**). (Please *see* the companion CD for the color version of this figure.)

cell differentiation into neuronal and epithelial lineages. Comparison of morphology and immunostaining of cells cultured in different media in a two-dimensional system reveals that ESCs predominantly differentiate into the neural lineage and slightly along the epithelial lineage in HPI.1-conditioned medium. In contrast, A5RT.1-conditioned medium effectively stimulates ESCs to differentiate along the epithelial lineage.

3.4.2. Culture of ESCs on Three-Dimensional Collagen Gel (see ***Note 8***)

ESCs cultured on three-dimensional collagen gel form dense clusters as well as tubular or spherical glandular-like structures, which became clearly evident after 19 d in culture (**Fig. 2E–H**). These cells are negative for stage-specific embryonic antigen 3 and 4. Some of these structures are formed by one cell layer (**Fig. 2E**), whereas others are formed by multiple cell layers surrounding a "lumen" (**Fig. 2F–H**) (we call these structures monolayered and multilayered circular structures, respectively).

We routinely stain sections with antibodies against cytokeratin for the epithelial lineage (**Fig. 2E**) and with nestin (**Fig. 2F**), NCAM (**Fig. 2G**), and class III β-tubulin (**Fig. 2H**) for the neural lineage. Other antibodies, under a similar protocol, can be used to characterize cell differentiation. The staining protocol is as follows:

1. Place paraffin-embedded slides in Citri-solv at room temperature for 10 min.
2. Rehydrate the slides by placing them sequentially in 100% ethanol for 6 min, in 95% ethanol for 6 min, and in distilled or deionized water for 5 min.
3. Perform target retrieval on all formaldehyde-fixed paraffin sections by heating the sections in target retrieval buffer in a microwave oven for 15 min.
4. Dilute antibodies in TBS plus 1% BSA.
5. Apply Vecton MOM IgG block reagents according to the manufacturer's instructions.
6. Follow the staining procedure for chamber slide cultures (*see* **Subheading 3.4.1**, **steps 5–7**).
7. Dehydrate the slides by placing them sequentially in 95% ethanol for 6 min, in 100% ethanol for 6 min, and in Citri-solv for 6 min.
8. Mount with xylene-based mounting medium.
9. Count positive-stained cells in nine unconnected fields at ×100 magnification and calculate the percentage of positive staining on the basis of the total number of cells in each view.

Under this protocol of ESCs grown in three-dimensional collagen gel, ESCs proliferate and differentiate into neural and epithelial lineages, forming circular structures resembling those evolving in embryogenesis. HPI.1 embedded in the collagen gel but not used as a feeder layer facilitates expression of the neural markers nestin, class III β-tubulin, and especially NCAM. In particular, in monolayered structures, the cytoplasms of the cells facing the lumen are more intensively stained with anti-cytokeratin antibodies than those of cells at the basal site (**Fig. 2E**). In multilayered structures, nestin-positive cells are located within various layers (**Fig. 2F**). NCAM-positive cells are confined to multilayered structures. Cells intensively stained for NCAM are located in the peripheral cell layers (**Fig. 2G**). As with NCAM staining, cells strongly expressing class III β-tubulin are predominantly located in multilayered structures, where they are found in the cell layers located farther from the lumen (**Fig. 2H**).

3.4.3. Culture of ESCs on Three-Dimensional Collagen Sponge (see ***Note 9***)

H&E staining reveals that, after 24 d in culture, some ESCs remain on the top of the sponge, whereas others penetrate into the pores and migrate downward (**Fig. 3A**). The cells within the pores tend to adhere to the collagen fibers and to form aggregates mostly in the upper and central parts of the sponge. Besides aggregates, single cells are scattered

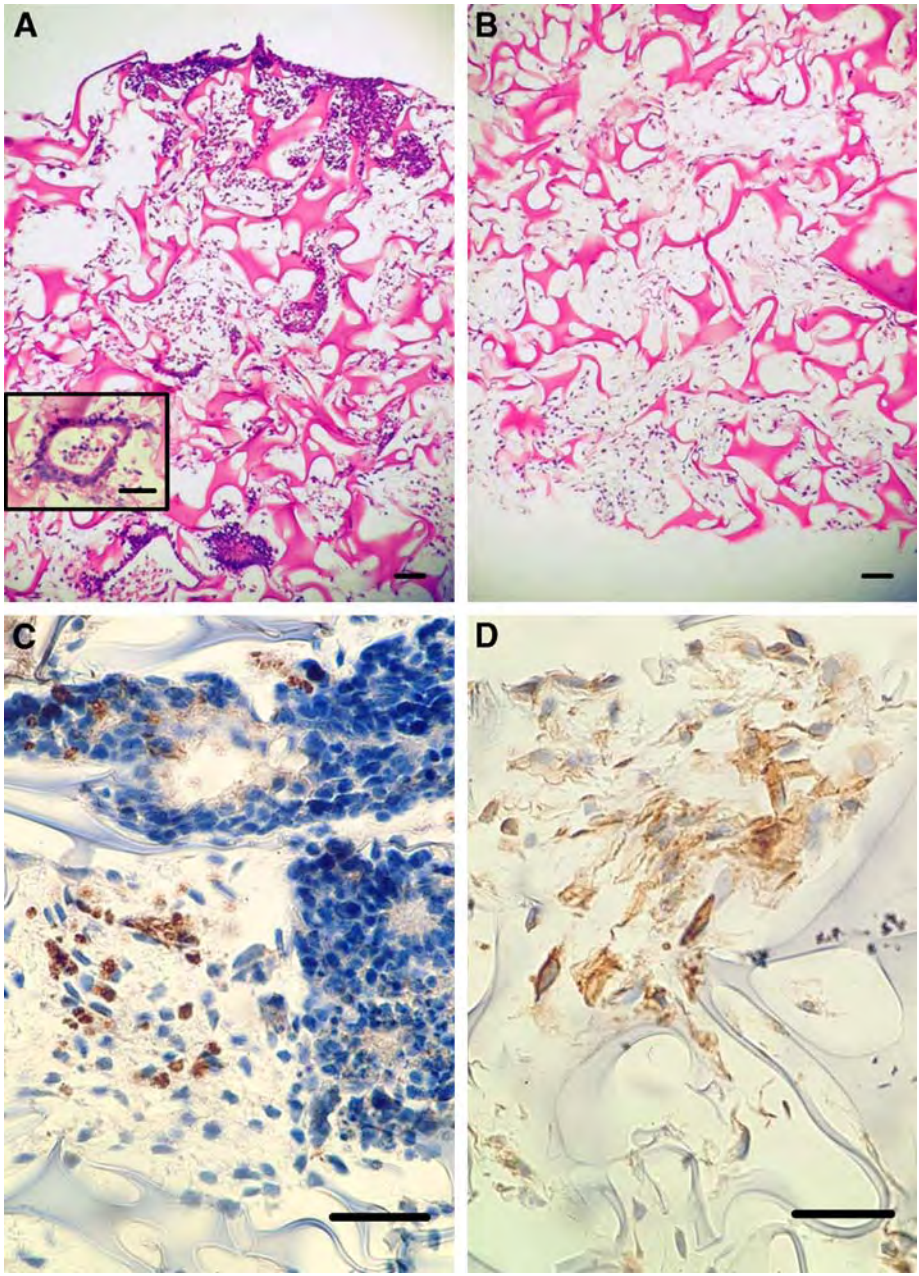

Fig. 3. Embryonic stem cells cultured in collagen sponge for 24 d. H&E staining (**A**,**B**) and immunohistochemical staining (**C**,**D**). Cells are stained for factor VIII (**C**) and vimentin (**D**) (*see* **Subheading 3.4.3.**). Scale bars = 50 µm. (Please *see* the companion CD for the color version of this figure.)

throughout the sponge (**Fig. 3B**), and the amounts of these cells gradually decrease from the top to the bottom. ESCs in collagen sponges form both monolayer and multilayer circular structures with a central lumen (**Fig. 3A** insets); however, the numbers of these structures formed within the sponge under this protocol are consistently lower than the numbers of structures formed in a collagen gel (*see* **Subheading 3.4.2.**).

To characterize cells in these cultures, we use a set of antibodies against cytokeratin, nestin, NCAM, chromogranin A, factor VIII (**Fig. 3C**), and vimentin (**Fig. 3D**). For the staining procedure, *see* **Subheading 3.4.2.** Factor VIII (**Fig. 3C**) is expressed in the cytoplasm of scattered single cells but not in cells within circular structures. The majority of vimentin-positive cells are among scattered single cells inside the sponge (**Fig. 3D**), whereas few of them are located in multilayer structures. Addition of GM-CSF to ESCs cultured with HPI.1 feeders does not affect ESC differentiation compared with ESCs grown with HPI.1 alone. In contrast, when GM-CSF is added to ESC cultures grown with A5RT.1 feeder, the expression of factor VIII can be further stimulated relative to the cultures grown with A5RT.1 alone. Thus, exogenous rhGM-CSF and the keratinocyte cell line that produces GM-CSF facilitate ESC differentiation into an endothelial lineage.

4. Notes

1. The mitomycin C solution should be stored at 4°C in the dark and used within 1 mo.
2. If it is a 1-to-3 split, then suspend one well of ESCs in a final volume of 6 mL ESC medium.
3. After treatment with collagenase medium, some colonies are still large. Resuspend the cells with a small volume of medium (about 2–3 mL) and pipet up and down several times until homogeneous suspension is observed. Then, add the rest of the medium until the target volume is reached.
4. We normally suspend one well of ESCs cultured in a six-well plate with 2 mL medium.
5. Collagen gel should be made on ice, and vigorous pipetting should be avoided; otherwise, air bubbles can be trapped inside the gel, and it is almost impossible to get rid of them later. Also, it is important to keep all the pipets and tools cold. We keep all the pipets and tools on ice while making the collagen gel.
6. The collagen sponge should be moisturized completely before use. Because dry collagen sponges are crisp and easily broken, they should be pushed gently with forceps in the medium until they become soft. Then, they should be pushed harder, until the color of the sponge changes from white to the color of the medium.
7. Avoid using xylene-based mounting medium on plastic chamber slides.
8. To characterize cells that have migrated into collagen gel, each piece of this gel should be cut tangentially into halves along the diameter and then embedded in paraffin and sectioned. Histological sections (approx 5 µm) have to be stained with H&E and subjected to immunohistochemical analysis.
9. For analysis of cells that have migrated inside and through the collagen sponge, each piece of it should be cut vertically into halves along the long axis, embedded in paraffin, and sectioned. Histological sections (approx 5 µm) should be stained with H&E or subjected to immunohistochemical analysis.

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