Springer Protocols

Kursad Turksen · *Editor*

Human Embryonic Stem Cell Protocols

Third Edition

Humana Press

METHODS IN MOLECULAR BIOLOGY

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Edited by

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Ottawa, ON, Canada

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Printed on acid-free paper

Preface

In spite of ethical and political concerns regarding the use of human embryonic stem cells, their potential to advance not only regenerative medicine applications but also our fundamental understanding of stem cell biology continues to drive interest in research with these cells. In both cases, delineating the steps in and identifying the molecules responsible for the commitment and differentiation of these pluripotent cells to different cell fates remains crucial. With this in mind, I felt that it would be timely to collect some of the most interesting and useful protocols that have emerged during the last several years. I am therefore extremely pleased and thankful to those groups who agreed to share their valuable protocols with others interested in exploring stem cell biology questions in a research setting.

Once again, I would like to acknowledge John Walker, Editor in Chief of the series, for continuously fostering my efforts in this series. I am also grateful to Patrick Marton, Executive Editor of the series, for encouraging me throughout the editorial process. In addition, I thank David Casey for keeping me on track and pointing out all those details that make contributions to the series consistent and dependable.

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Derivation of Human Embryonic Stem Cell Lines from Vitrified Human Blastocysts

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Abstract

Human embryonic stem cells are pluripotent cells typically derived from blastulating embryos that have become excess to clinical needs in assisted reproduction programs. They provide cellular models for embryonic development and disease, and are thought to be useful for future cell replacement therapies and regenerative medicine. Here we describe methods to derive human embryonic stem cell lines. This includes blastocyst cryopreservation using a highly efficient vitrification protocol, the production and use of fibroblast feeder cells, embryo plating and passaging of resulting cellular outgrowths, and cryopreservation of putative stem cells lines.

Keywords: Blastocyst, Cryopreservation, Derivation, Embryonic stem cell, Fibroblast feeder cells, Human, Vitrification

1 Introduction

Human embryonic stem cells (hESCs) were first described by Thomson and colleagues in 1988 (1) and are most commonly derived from the pluripotent inner cell mass (ICM) of blastulating embryos. The source of donated embryos is typically excess blastocysts from assisted reproduction programs, whether because a couple has completed their family or because the embryo was found to be clinically unsuitable for transfer due to severe genetic condition. Their unique characteristics include the ability to grow in vitro indefinitely and the capacity to differentiate into specialized somatic cell types from the three germ layers. This makes them and their differentiated products valuable in vitro models of embryonic development and disease, as well as tools for drug discovery and toxicity screening (2). hESCs are also widely considered to be clinically useful for still to be perfected cell replacement therapies and regenerative medicine (3).

This chapter covers the steps from the moment a human embryo is assigned for cryostorage, to be later possibly used for

stem cell derivation, up until a new hESC line has been generated and secured by cryopreservation. This includes embryo cryopreservation using a highly efficient modified Cryotop vitrification method (4), the production and mitotic inactivation of primary fibroblast cells used as a feeder layer for hESCs, and derivation of putative hESC lines including embryo plating, initial embryonic outgrowth passages, and cryopreservation. The methods for human IVF are not described, nor are the methods to expand and characterize the putative hESC line once obtained, as they fall outside the aims of this chapter.

2 Materials

2.1 Embryo Vitrification and Warming

1. Biological safety cabinet.
2. Stereomicroscope with heating stage.
3. Embryo incubator (K-MINC-1000 mini-incubator, Cook IVF, Cat No. K-MINC-1000).
4. CVM Vitrification Block with handle and lid (Cryologic, Cat No. CVB, Victoria, Australia). The block should be sterilized using dry heat 140 °C for 6 h or 160 °C for 2 h.
5. CVM Cryobath with lid (Cryologic, Cat No. LNB).
6. Cryotop vitrification device (Kitazato, Cat No. 81111 to 81115, Japan).
7. 4-well plates (Nunc, Cat No. 144444).
8. Indirect mouth hose (preventing open airway contact with solution) and embryo handling pipettes. Alternatively, hand-held micromanipulation pipettes can be used (Flexipet, Cook IVF, Cat No. K-MPH-1000).
9. P2, P20, P200, and P1000 pipettes and tips.
10. Liquid nitrogen storage dewar, canes, and goblets.
11. Sydney IVF Blastocyst Vitrification Kit (Cook IVF, Cat No. K-SIBV-5000) (*see* **Note 1**) consisting of a Solution 1 (Cryobase buffer), Solution 2 (used during vitrification after addition of DMSO to make a final solution of 8 % ethylene glycol and 8 % DMSO in Cryobase), Solution 3 (used during vitrification after addition of DMSO to make a final solution of 0.68 M trehalose, 16 % ethylene glycol, and 16 % DMSO in Cryobase) and Solution 4 (DMSO).
12. Blastocyst warming solutions: 1) Cryobase, 2) 1 M trehalose in Cryobase and 3) 0.5 M trehalose in Cryobase. Cryobase is HEPES buffered medium containing 20 mg/mL human serum albumin and 0.01 mg/mL gentamicin (Cook IVF).

13. Sydney IVF Blastocyst Medium (Cook IVF, Cat No. K-SIBM-20) (*see* **Note 1**).

14. Embryo culture grade oil (COOK IVF, Cat No. K-SICO-200).

15. Liquid nitrogen.

2.2 Fibroblast Feeder Cell Preparation

1. Class II biological safety cabinet.

2. Cell culture incubator.

3. Microscope.

4. Gamma irradiation device.

5. Liquid nitrogen dewar with box storage.

6. Liquid waste container or aspirator system.

7. P2, P20, P200, and P1000 pipettes and tips.

8. Cryo label printer and cryo labels or a permanent marker.

9. Forceps and dissecting scissors.

10. Scalpel blades and scalpel blade handle.

11. Cell counter.

12. 0.22 μm sterile filters.

13. 1, 5, 10, 25, and 50 mL pipettes.

14. 15 and 50 mL centrifuge tubes.

15. 1.2 mL cryovials.

16. Culture vessels, e.g., 1-well organ culture dishes (BD Falcon, Cat No. 353037), 4- or 6-well plates (Nunc, Cat No. 176740 and 140675, respectively), and T25, T75, or T175 flasks (BD Falcon, Cat No. 353014, 353024, and 353028, respectively).

17. Human tissue sample, aseptically and freshly collected, preferably at least a few grams (or approximately 5×5 mm in size). Alternatively, an established commercially sourced feeder cell line can be used (e.g., CCL-110 human fibroblast line, ATCC).

18. Feeder medium consisting of Dulbecco's Modified Eagle's Medium (DMEM high glucose, no L-glutamine, no sodium pyruvate, Life Technologies, Gibco, Cat No. 11960-044) supplemented with 10 % fetal calf serum (Cat No. 16141-079), $1\times$ MEM nonessential amino acids (Cat No. 11140-050), 2 mM Glutamine (Cat No. 25030-149), 1 mM Sodium Pyruvate (Cat No. 11360-070), and 50 U/mL penicillin and 50 μg/mL streptomycin (Cat No. 15140-122). The medium is sterile filtered and stored at +4 °C for a maximum of 1 month.

19. Magnesium and calcium-free PBS (PBS (−)).

20. 0.25 % trypsin.

21. 0.1 % gelatin (Millipore, Cat No. ES-006-B).

22. DMSO.

23. 70 % ethanol.

2.3 hESC Derivation	1. Class II biological safety cabinet.

1. Class II biological safety cabinet.

2. Stereomicroscope with heating stage and eyepiece extension making it suitable for working inside a class II biological safety cabinet.

3. Cell culture incubator (or embryo incubator).

4. Indirect mouth hose (preventing open airway contact with solution) and embryo handling pipettes. Alternatively, hand-held micromanipulation pipettes can be used (Flexipet, Cook IVF, Cat No. K-MPH-1000).

5. P2, P20, P200, and P1000 pipette and tips.

6. Ultra sharp splitting blades (e.g., Bioniche Animal Health, Cat No. ESE020), washed and sterilized. These can be used several times until becoming blunt.

7. Mitotically inactivated feeder cell plate (see Section 3.2, plated 1–7 days prior to use) with hESC medium.

8. Blastocyst culture medium (Cook IVF, Cat No. K-SIBM-20).

9. Embryo handling medium (Cook IVF, Cat No. K-SIFB-100).

10. Embryo culture grade oil (Cook IVF, Cat No. K-SICO-200).

11. hESC culture medium consisting of Knock Out-DMEM (Life Technologies, Gibco, Cat No. 10829-018) supplemented with 2 mM glutamine (Cat No. 25030-081), 50 U/mL penicillin and 50 μg/mL streptomycin (Cat No. 15140-122), 1× MEM nonessential amino acids (Cat No. 11140-050), 0.1 mM β-mercaptoethanol (Cat No. 21985-023), and 20 % Knock-out Serum Replacement (Cat No. 10828-028). The medium is sterile filtered and stored at +4 °C for a maximum of 1 month. Just prior to use add bFGF (Chemicon, Cat No. GF-003) to a final concentration of 20 ng/mL.

12. 70 % ethanol.

2.4 Vitrification and Warming of Putative Early hESCs

1. Class II biological safety cabinet.

2. Stereomicroscope with heating stage and eyepiece extension making it suitable for working inside a class II biological safety cabinet.

3. Cell culture incubator.

4. Liquid nitrogen storage dewar, canes, and goblets.

5. P20, P200, and P1000 pipette and tips.

6. Cryo label printer, cryo labels, and permanent marker.

7. Open pulled straws (OPS), 0.5 mL (outer) straws, and straw putty.

8. 4-well plates (Nunc, Cat No. 144444).

9. 0.22 μm filters.

10. Sterile forceps.

11. Ultra sharp splitting blades (e.g., Bioniche Animal Health, Cat No. ESE020), washed and sterilized. These can be used several times until becoming blunt.

12. Mitotically inactivated feeder cell plates (see Section 3.2, plated 1–7 days prior to use) with hESC medium.

13. 2 M sucrose solution in DMEM-HEPES.

14. DMEM-HEPES (without phenol red).

15. Fetal calf serum (FCS).

16. DMSO.

17. Ethylene glycol.

18. Liquid nitrogen.

3   Methods

3.1   Embryo Vitrification and Warming

Vitrification is a method of cryopreservation which uses a high concentration of cryoprotectants and ultra-rapid cooling to freeze cells in a glass-like state. Unlike conventional slow freezing, vitrification avoids detrimental ice crystal formation resulting in excellent (>90 %) survival rates after embryo thawing. However, prolonged exposure to high concentrations of cryoprotectants increases embryotoxicity, making vitrification as demanding as it is successful when properly executed. The method described here utilizes solutions from the Sydney IVF Blastocyst Vitrification Kit (*see* **Note 1**) with a modified Cryotop protocol whereby vitrification occurs on the Cryotop device by contact with a metal block precooled with liquid nitrogen. This procedure minimizes embryo contact with liquid nitrogen to prevent potential cross-contamination and has been found to result in pregnancy rates upon warming equivalent to that of fresh embryo transfers (4).

All protocols involving embryos should be performed in a biological safety cabinet under sterile conditions using aseptic techniques and preferably disposable consumables. Embryo incubation should be performed in a controlled humidified atmosphere of 6 % CO_2, 5 % O_2, and 89 % N_2 at 37 °C, preferably in a mini-incubator with separately controlled culture chambers, and embryos should be removed from these conditions only for a minimum length of time required for a given procedure.

3.1.1   Embryo Vitrification

1. Appropriately label top and bottom of 4-well dish(es). One dish can be used to vitrify two embryos from a single patient. Prepare each vitrification dish as follows using solutions from the Sydney IVF Blastocyst Vitrification Kit (*see* **Note 1**):

(a) To well #1 and #2 add 460 μL Solution 2 and 40 μL Solution 4 and mix thoroughly.

(b) To well #3 and #4 add 420 μL Solution 3 and 80 μL Solution 4 and mix thoroughly.

(c) Allow the dish to come to room temperature for 10 min and use within 60 min.

2. Appropriately label Cryotop vitrification device(s) (one for each embryo to be vitrified), as well as cane(s) for liquid nitrogen storage.

3. Fill the cryobath with liquid nitrogen to approximately half the height of the metal vitrification block. Slowly lower the metal block with lid into the bath and allow cooling for at least 5 min before adding further liquid nitrogen to the level just below the top of the block.

4. Fill a liquid nitrogen container with liquid nitrogen and place the labeled cane(s) into the bucket to precool.

5. Place Cryotop sheath(s) into the metal block for cooling prior to use.

6. Move a single embryo to the top of the medium in well #1. Observe the embryo for collapse of the blastocoel cavity (*see* **Note 2**) and subsequent re-expansion. The embryo is ready to be moved to the next solution after 12 mins if the embryo has expanded to >80 % of its initial volume and has not exhibited any further expansion within a 1 min timeframe, or if 15 min has expired.

7. If freezing an additional embryo this may be transferred to well #2 of the vitrification dish 3–5 min after the first embryo. Additional embryos from the same patient can be transferred to additional vitrification dishes at 3–5 min intervals.

8. The following steps from 9 to 12 should be completed in 60–90 s.

9. When equilibration is complete, transfer the embryo in a minimal volume to the surface of solution in well #3. A swirling motion whilst dispelling the embryo can aid in the removal of the previous solution. Wash the embryo thoroughly by moving it around the well.

10. Transfer embryo in a minimal volume to the top of the solution in well #4. Wash the embryo as per step 9.

11. Transfer the embryo in a minimal volume from well #4 onto the Cryotop device (logo side up) near the black marking. Aspirate excess solution with pipette (*see* **Note 3**).

12. Transfer the Cryotop device to the vitrification block and place the end of the Cryotop onto an allocated spot on the block surface for 5–7 s.

13. Quickly insert the Cryotop device into a chilled sheath and gently press down to seal. During this process keep the Cryotop as close as possible to the metal block to avoid warming.

14. Move Cryotop to the labeled cane in liquid nitrogen.

15. Repeat steps 6 onwards for remaining embryos. When all embryos from the patient have been vitrified they can be transferred to long-term liquid nitrogen storage. This should be done as quickly as possible to avoid temperature fluctuations (*see* **Note 4**).

3.1.2 Embryo Warming

1. At least 4 h or overnight prior to warming, prepare blastocyst culture dish(es) containing drops of Sydney IVF Blastocyst Medium (*see* **Note 1**) covered with equilibrated mineral oil, and incubate at 37 °C with 6 % CO_2. Also include a "wash" well containing 500 μL of Blastocyst Medium.

2. Prepare one 35 mm dish per patient containing 2.5 mL of 1 M trehalose in Cryobase and warm to 37 °C for at least 10 min.

3. Prepare one 4-well dish per patient as follows and allow to come to room temperature:

    (a) To well #1 add 500 μL 0.5 M trehalose in Cryobase.

    (b) To well #3 and #4 add 500 μL Cryobase.

4. Transfer Cryotop device containing embryo to be thawed to a liquid nitrogen container and place next to microscope.

5. Focus the microscope (warming stage off) view on the bottom of the 35 mm dish containing 1 M trehalose in Cryobase.

6. Using tweezers with the tips cooled in liquid nitrogen, hold the Cryotop protective cover under liquid nitrogen and twist Cryotop to loosen. Extract Cryotop, using care not to contact the liquid nitrogen, and immediately place end into the 35 mm dish, stirring it in a small circle until the embryo falls off the Cryotop (*see* **Note 5**).

7. After 1 min, quickly move the embryo to the bottom of well #1 of the 4-well dish. Leave the embryo undisturbed (even if it floats to the surface) for 3 min.

8. Transfer the embryo to the bottom of well #3 of the 4-well dish. Leave embryo undisturbed for 5 min.

9. Transfer the embryo to well #4 of the 4-well dish. Leave embryo undisturbed for 1 min.

10. Transfer the embryo to the wash well of the prepared blastocyst culture dish and move around gently to remove any remaining warming solution.

11. Transfer the warmed embryo into culture drop in the prepared blastocyst culture plate, place in embryo incubator, and allow

to recover for at least a few hours prior to hESC derivation. If desired, the embryo can be left overnight before plating. However, this has to be determined on a case by case basis, depending on the appearance and developmental stage of the embryo.

3.2 Fibroblast Feeder Cell Preparation

hESCs are commonly cultured on a layer of primary fibroblast cells, the so-called "feeder cells," to promote hESCs attachment and provide factors important for proliferation and pluripotency. Although it is possible to culture hESCs on semi-defined extracellular matrices, the high demands of deriving a new hESC line (supporting the proliferation of a small number of pluripotent cells within the embryo) reduce the temptation of using these matrices during derivation attempts. Feeder cells should ideally be from human origin and can be initiated from primary tissue samples such as neonatal foreskin or from commercially sourced established primary cell lines. Whether using commercial or in-house prepared feeders, it is advisable to prepare and freeze "master stocks" of early passage cells to serve as a starting point for subsequent expansion of "working stocks" for mitotic inactivation. Mitotic inactivation of feeder cells is essential prior to derivation and culture of hESCs and can be achieved efficiently and conveniently by gamma irradiation as reported here, or by Mitomycin C treatment as described previously (5).

All protocols involving feeder cell culture should be performed in a biological safety cabinet under sterile conditions using aseptic techniques and preferably disposable consumables. Incubation of feeder cell cultures should be performed in a controlled humidified atmosphere of 5 % CO_2 in air at 37 °C.

3.2.1 Establishing a Primary Feeder Cell Line

1. Obtain the sample of human tissue (*see* **Note 6**) using sterile techniques and proceed with processing as soon as possible.

2. Transfer the tissue using sterile forceps to a 35 mm Petri dish containing 3–4 mL of PBS(−) (*see* **Note 7**).

3. Agitate the dish gently to wash the tissue, removing adhering blood or mucus if possible.

4. Repeat steps 2 and 3 until satisfied the tissue is as clean as possible (usually 2–4 washes), then transfer the tissue to a new empty dish (the tissue should still be moist with PBS).

5. Finely cut the tissue into pieces of at least 1 mm^2 or as small as possible using a scalpel blade. Ideally the pieces should be of similar size so that attachment will occur at the same rate.

6. Transfer a minimum of 6–8 small pieces of tissue (*see* **Note 8**) to the bottom of an empty T25 cell culture flask lying horizontally. Replace the cap on the flask and stand upright for 15–20 min (*see* **Note 9**). Prepare multiple flasks this way to ensure enough

material for the cultures and to safeguard against accidental contamination or other loss of a single flask.

7. Add 5 mL of warm feeder medium to the bottom of each flask in an upright position taking care to avoid the attached tissue pieces.

8. Gently tilt the flask until the first tissue pieces touch the medium and if they remain attached continue tilting until the flask lies in a horizontal position. Carefully transfer the flask into the incubator avoiding movement that may dislodge the pieces. Alternatively, the upright flasks can be transferred to the incubator immediately after adding the medium and then tilted in the incubator to minimize movement.

9. If the first tissue pieces detach upon contact with the medium, return the flask to the upright position and allow the remaining pieces to dry for another 5 min before attempting to tilt the flask. Repeat as necessary. Having just a few pieces detaching and floating is not detrimental to the subsequent culture, but if the source material is scarce these pieces can be removed from the flask, transferred to an empty flask, and the attachment procedure repeated.

10. Flasks should not be disturbed for a minimum of 48 h, after which they can be assessed under a microscope for the outgrowth of fibroblast cells from the attached tissue piece. Depending on the origin of the tissue, other cell types may also be observed such as epithelial cells.

11. If the tissue pieces remain attached and outgrowth of cells is visible, replace the culture medium with fresh medium to remove any free floating pieces, red blood cells, or debris. If the tissue pieces have not attached and the source tissue is scarce, it may be worthwhile to attempt reattachment using the procedure described above.

12. The medium should be changed on the flasks approximately every 6 days until they are ready for passaging for further expansion or cryopreservation. This occurs when the cells originating from the attached tissue pieces are 70–80 % confluent and still in the exponential growth phase, which may take approximately 5–10 days (from plating) depending on the amount of tissue plated.

3.2.2 Passaging Feeder Cells

1. Examine the cultures under a microscope to determine the split ratio. For expanding fibroblast feeder cells this is usually between 1:2 and 1:5 (i.e., one flask of cells split to two to five new flasks).

2. Warm the required volume of PBS(−), 0.25 % trypsin, and feeder medium for passaging and ongoing culture (see Table 1;

Table 1
Passaging of human fibroblast feeder cells

Vessel	PBS(−)	0.25 % trypsin	Feeder medium (passaging)	Feeder medium (culturing)
T25	3	1	4	7
T75	10	2	8	20
T175	20	4	16	50

Volumes (mL) of PBS, 0.25 % trypsin and feeder medium required for cell passaging for commonly used culture vessels

note that feeder medium is also required during passaging for trypsin inactivation).

3. Transfer required reagents and consumables to a biological safety cabinet followed by the feeder flask(s) to be passaged. Ideally no more than 4 flasks should be passaged simultaneously (*see* **Note 10**).

4. Aspirate medium from flask(s) to a waste container and add PBS(−) to wash the cells (see Table 1).

5. Aspirate PBS(−), add 0.25 % trypsin (see Table 1), and gently agitate to evenly distribute.

6. Incubate the cells in trypsin at 37 °C for 2–3 min then tap the side of flask(s) several times to dislodge the cells. Ensure the cells have completely detached by visualizing under a microscope.

7. Once cells have detached, add approximately 4× volume of feeder medium (see Table 1) to the flask(s) and mix. Pipette the cells several times and transfer to a 50 mL tube.

8. Collect any remaining cells in the flask by addition of new feeder medium (this same volume can be transferred into each flask sequentially) and add to the 50 mL tube. The cells from several flasks can be combined to one or more tubes depending on the number of flask processed.

9. Centrifuge the tube(s) for 4 min at $300 \times g$.

10. Remove supernatant being careful not to disrupt the cell pellet.

11. Resuspend cells in an appropriate volume of feeder medium depending on the number of flasks to be seeded. This can be calculated on the basis of 1–2 mL of cell suspension for each new flask, or alternatively, if splitting cells to only a few flasks, the cells can be resuspended in the final volume of feeder medium (see Table 1).

12. Pipette the required volume of cell suspension to each new flask and if required add feeder medium to achieve the final culture volume.

13. Transfer newly plated flasks into incubator until ready for further passaging or freezing. Generally media change is not required during this time (*see* **Note 11**) unless culturing for longer than 7–8 days.

3.2.3 Freezing Feeder Cells

1. Fibroblast feeder cells are ready to be frozen when they have reached 70–80 % confluence and are still in an exponential growing phase. Typically cells are frozen at a concentration of 1×10^6/mL in 0.5 mL or 1 mL aliquots. This may have to be performed in several batches depending on the number of flasks (and cells) to be frozen (*see* **Note 12**).

2. In preparation for freezing, label the estimated number of cryovials with cell line details and passage number, the freezing date, and the number of cells for cryopreservation (*see* **Note 13**). Place cryovials in a tube rack and cool by storing at $-20\,^{\circ}$C until required.

3. Trypsinize the cells as per passaging protocol (steps 2–10 in Section 3.2.2).

4. After centrifugation, resuspend cells in a suitable volume of room temperature feeder medium to enable accurate counting. For example, 1–3 mL per T75 flask containing 70–80 % confluent cells if using a hemocytometer.

5. Determine the total number of viable cells in the cell suspension.

6. Calculate the volume required to dilute cells to the desired concentration taking into account the volume of feeder medium in which the cells are currently suspended and that 10 % of the final volume is to consist of DMSO (*see* **Note 14**).

7. Add the remaining volume of feeder medium to the cell suspension, mix gently, and place tube on ice.

8. Retrieve the tube rack containing labeled cryovials from the freezer and loosen the lids in preparation for use.

9. Add required volume of DMSO to the tube containing the cell suspension so that the final freezing medium composition is 90 % feeder medium and 10 % DMSO. Mix thoroughly keeping the tube on ice.

10. Pipette the appropriate volume of freeze medium containing resuspended cells into each cryovial, mixing the remaining cells in the 50 mL tube frequently to ensure that the aliquoted cell numbers remain consistent throughout the process. Work quickly keeping the cell mixture on ice and the cryovials in

the precooled rack and handling them as little as possible to minimize warming.

11. Quickly transfer the cryovials into a −80 °C freezer overnight or maximum for a few days. Long-term storage at −80 °C is not recommended as viability can be compromised.

12. Quickly transfer the cryovials from the −80 °C freezer into liquid nitrogen storage. This should be done in a way that ensures that harmful warming of the vials does not occur.

3.2.4 Thawing Feeder Cells

1. Determine the number of vials to be thawed. Depending on the fibroblast feeder cell line, the plating density for further expansion can be as low as 5,000 cells per cm^2 surface area.

2. In preparation for thawing, fill a suitable container with 37–40 °C water or set up a water bath to this temperature range.

3. Remove the cryovials(s) from liquid nitrogen storage and transfer to the laboratory in a suitable container filled with liquid nitrogen, dry ice or nothing, depending on time until processing; cryovial(s) can be transported at room temperature for a minute if processed immediately. Depending on the number of cyrovials required, thawing may have to be performed in several batches (*see* **Note 15**).

4. Pipette a minimum of 7 mL of warm feeder medium per every 0.5 mL of frozen cells to be thawed into a 50 mL tube.

5. Place the cryovials(s) into warm water and thaw until only a sliver of ice can be seen in the vial. This usually takes a few minutes and is faster if the vial is moved through the water.

6. Quickly clean the outside of the cryovial(s) with 70 % ethanol and transfer to the biological safety cabinet.

7. Pipette the contents of the vial(s) slowly and drop-wise into the 50 mL tube containing feeder medium.

8. Pipette 1 mL of feeder medium containing thawed cells back to the cryovial(s) to recover any remaining cells then transfer to the 50 mL tube. Depending on the number of vials thawed, the cells from several vials can be collected to one or more 50 mL tubes.

9. Centrifuge the tube(s) for 4 min at 300 × *g*.

10. Remove supernatant being careful not to disrupt the cell pellet.

11. Resuspend cells in an appropriate volume of feeder medium depending on the number of flasks to be seeded. This can be calculated on the basis of 1–2 mL of cell suspension for each new flask, or alternatively, if plating cells to only a few flasks, the cells can be resuspended in the final volume of feeder medium (see Table 1).

12. Pipette the required volume of cell suspension to each new flask and if required add feeder medium to achieve the final culture volume.

13. Transfer newly plated flasks into incubator until ready for further passaging or mitotic inactivation. Generally media change is not required during this time (*see* **Note 11**) unless culturing for longer than 7–8 days.

3.2.5 Mitotic Inactivation of Feeder Cells by Gamma Irradiation

1. Fibroblast feeder cells are ready for gamma irradiation when approaching confluence (approximately 90 %). We typically prepare a batch of 100–150 million feeder cells for gamma irradiation (16 × T175 flasks) and cryopreservation.

2. Harvest the cells as described for feeder cell passaging (steps 2–10 in Section 3.2.2).

3. After centrifugation, resuspend the pelleted cells in feeder medium and transfer tubes to ice.

4. Irradiate cell suspension with 4,000 rad gamma irradiation.

5. Determine concentration of viable cells and freeze working stocks of mitotically inactivated feeder cells at 1×10^6/mL as described in Section 3.2.3 (steps 6–11). Mitotically inactivated feeder cells can also be plated immediately after irradiation for hESC derivation and culture the following day.

6. After preparation of a batch of gamma irradiated feeder cells, it is sensible to perform quality control measures to determine success of mitotic inactivation (*see* **Note 16**).

3.2.6 Plating of Mitotically Inactive Feeder Cells

1. Coat the surface of the required culture vessels with 0.1 % gelatin (see Table 2) and incubate at room temperature for a minimum of 10 min (and up to several hours).

2. Aspirate 0.1 % gelatin from vessels and allow to dry in a biological safety cabinet.

3. If using organ culture dishes or 4-well plates, add PBS(−) to the moat of the dishes or to the centre of the plates (*see* **Note 17**).

4. Determine required number of mitotically inactivated feeder cells (see Table 2 and **Note 18**).

5. Thaw vials of mitotically inactivated feeder cells as per Section 3.2.4 (steps 2–10).

6. Resuspend the cell pellet in a suitable volume of feeder medium and determine number of viable cells.

7. Pipette the required amount of cell suspension to each vessel and if required, make up final volume with feeder medium (see Table 2).

Table 2
Preparation of mitotically inactivated fibroblast feeder cell vessels for hESC culture

| Vessel | Growth area (cm^2) | Gelatin (mL) | Feeder cell | | Medium (mL) |
			Density	# ($\times 10^6$)	
4-well plate	1.9	0.2	High	0.13	0.7
			Low	0.045	
Organ culture dish	2.9	0.3	High	0.2	1
			Low	0.07	
6-well plate	9.5	1	Low	0.23	3
35 mm dish	10	1	Low	0.24	3
T25 flask	25	2	Low	0.6	7
T75 flask	75	5	Low	1.8	20

Volumes of 0.1 % gelatin and medium, and number of mitotically inactivated fibroblast cells for commonly used culture vessels. A higher density of feeder cells is used when deriving hESC lines and a lower density when culturing established hESC lines. Values given for 4-well and 6-well plates are for a single well

8. Transfer culture vessels to incubator. The mitotically inactivated feeder cell cultures are ready for use the following day, and no later than 3 days after plating for hESC derivation and 7 days for established hESC lines.

3.3 hESC Derivation   hESC lines are typically derived from excess cryopreserved blastocysts created for assisted conception. The scarcity of donated human embryos has meant that the methods for hESC derivation have not changed significantly since the first reports of primate embryonic stem cell generation (6) and still rely on cultivation on top of a fibroblast feeder cell layer and mechanical passaging. This section describes the techniques of hESC line derivation including embryo zona pellucida removal, bisection, and plating, which are followed by first critical manual passages of early embryonic outgrowths. These methods have been used for successful derivation of over 100 hESC lines (see www.geneabiocells.com), including GMP-level clinical grade lines (7), PGD-derived lines (8, 9), and lines derived from poor quality clinically unsuitable embryos (10).

All protocols involving embryo and cell culture work should be performed in a biological safety cabinet under sterile conditions using aseptic techniques and preferably disposable consumables. Cell culture incubation should be performed in a controlled humidified atmosphere of 5 % CO_2 in air at 37 °C or, preferably, in 6 % CO_2, 5 % O_2, and 89 % N_2 at 37 °C.

3.3.1 Embryo Plating

1. At least one day prior to embryo plating (and no more than 3 days) prepare mitotically inactivated fibroblast feeder dish(es) as described in Section 3.2.6. The most practical vessel for hESC derivation is the 1-well organ culture dish as it allows reasonable access for manipulation.

2. Replace the feeder medium in the feeder dish with warm gas-equilibrated hESC medium (*see* **Note 19**) and return to incubator until required.

3. Assess the donated embryo microscopically and confirm that it is suitable for plating (*see* **Note 20**). Determine if the embryo needs to be released from the zona pellucida and, depending on embryo quality and developmental stage, if bisection should be performed (*see* **Note 21**).

4. If the embryo is still enclosed inside the zona pellucida, it can be released mechanically with a cutting blade (*see* **Note 22**).

   (a) In a 35 mm Petri dish containing 3 mL embryo handling medium draw several parallel grooves in the bottom of the dish close together (10–20 μM) using a sterile cutting blade. These grooves will be used to secure the embryo during cutting.

   (b) Transfer the embryo to this dish (*see* **Note 23**) and move the embryo until it lies on top of the grooves and the ICM is visible.

   (c) Press the tip of the blade against the bottom of the dish slightly beyond the embryo and very gently press the rest of the blade down directly on top of it. The zona pellucida will not be cut cleanly at this point due to its elasticity and resistance, but it will flatten. Once clearly flattened, move the blade carefully sideways, aiming to roll the embryo slightly to its side, positioning the blade so that it is pressing down diagonally on the zona. Keep the tip of the blade gently pressed at the bottom of the dish at all times to give it necessary support. Then press the blade down completely, slicing a piece of zona. Always do the cut well away from the ICM and preferably in the area where the space between the zona and the embryo is the widest.

   (d) After the zona has been sliced open, if the opening is wide enough the embryo may be released by gentle agitation with a pipette or by applying gentle pressure using the blade. However, often it is necessary to cut the zona several times in a similar manner to widen to opening.

   (e) Remove any remaining zona remnants from the embryo by gentle pipetting.
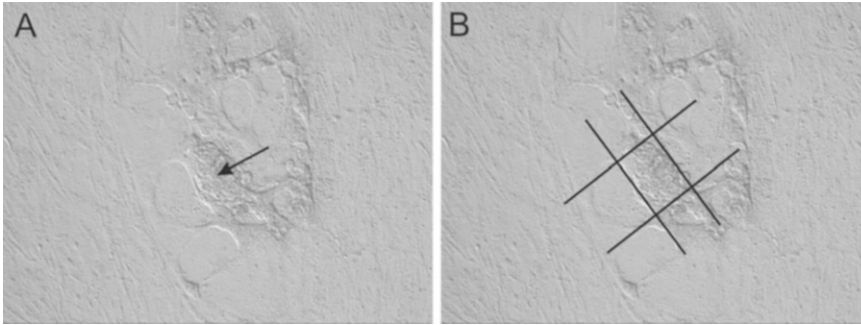
Fig. 1 Early embryonic outgrowth originating from plated blastocyst. (**a**) ICM-derived "bud" (*arrow*) surrounded by TE-derived cells and (**b**) *cutting lines* indicating where bud will be excised for first passage

5. Once the embryo has been released from the zona pellucida, if desired it can be bisected to isolate the ICM (with polar trophectoderm). This can be performed over grooves as per mechanical zona removal or on a flat surface at the bottom of the dish.

   (a) If zona pellucida removal was not required (and thus the embryo remains in the culture plate, e.g., hatched embryos), transfer to a 35 mm Petri dish containing 3 mL embryo handling medium (*see* **Note 23**).

   (b) Using a sterile cutting blade bisect the embryo either in the middle or asymmetrically, cutting as close as possible to the ICM.

   (c) Release the embryo half containing ICM from the bottom of the dish by gentle agitation or if necessary, by carefully using a blade or gentle pipetting. If this fails, use a fire-polished glass pipette and very gently tease the embryo half away from the plate.

6. Transfer the whole embryo or bisected embryo half containing the ICM (*see* **Note 24**) to a feeder dish and move to the incubator (*see* **Note 25**).

7. Change the media in the plates every second day until ready for passaging.

3.3.2 Passaging Early Embryonic Outgrowths

1. Observe plated embryo(s) for epiblast-like outgrowth every day or every second day after plating to determine the optimal time for passaging, typically between 5 and 8 days after embryo plating. This will depend on several factors, the most critical being the abundance and location of trophectodermal (TE)-derived cells surrounding the ICM-derived outgrowth or "bud" (*see* **Notes 26** and **27**). Ideally passaging will occur when the bud is easily distinguished, compact, and consisting of 100 or more cells, and is still separated from TE-derived cells (see Fig. 1a).
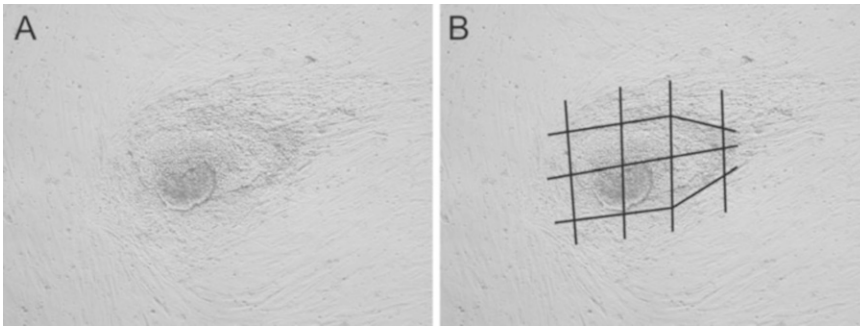
Fig. 2 Early manifestation of a putative hESC line. (**a**) Putative hESC colony emerging from passaged embryonic outgrowth and (**b**) *cutting lines* indicating fragments to be excised for second passage. Note that sections of hESC colonies containing differentiated cells should not be passaged

2. At least one day prior to passaging, prepare mitotically inactivated fibroblast feeder dish(es) as described in Section 3.2.6. On the day of passaging, replace the feeder medium with warm gas-equilibrated hESC medium and return to incubator until required.

3. Cut the bud away from the surrounding TE-derived cells and feeder cells (see Fig. 1b). This is ideally performed with a cutting blade although it is also possible to use a sharp-edged glass pipette. If a clear bud is not visible even after 8–10 days after embryo plating proceed with step 5.

4. Tease the bud away from the plate using a pipette and transfer to the fresh feeder dish containing hESC medium.

5. Cut any remaining outgrowth of epiblast origin into a few pieces and distribute to the same feeder dish evenly and away from the original bud.

6. Transfer the newly passaged (now passage 1) outgrowth(s) to the incubator along with the original plating dish to serve as a backup.

7. Change hESC medium every second day until ready for passaging (approximately 7 days). At this stage it should be possible to see putative hESCs growing out of the plated outgrowth(s), and possibly even colony morphology typical of hESC lines (Fig. 2a), although putative hESC colonies are often only clearly visible in passage 2–3 cultures. TE-derived cells may also be present but will be significantly reduced from the original embryo plating.

8. Manually passage outgrowth(s) as described above (now passage 2), either as a single piece or several small fragments depending on appearance and size (see Fig. 2b), and return to incubator with media change every second day.

9. Continue manual passaging and culturing until an early hESC line has been established. It is advisable to cryopreserve an putative hESC line as soon as possible (ideally passage 3–5) and is worthwhile to vitrify even the smallest fragment of colonies to ensure the survival of the line in case of loss of culture.

3.4 Vitrification and Warming of Putative Early hESCs

Unlike fibroblast feeder cells and mouse embryonic stem cells, hESCs should not be slow frozen in single cell suspension but vitrified as small cell clumps or fragments. This cell vitrification method follows the same principle as embryo vitrification and greatly enhances the survival rate of warmed hESC lines. The protocol involves the stepwise introduction of the cells into the vitrification solution with a high concentration of cryoprotectants DMSO and ethylene glycol together with sucrose to increase the osmolality of the solution. Cell fragments are then loaded into open pulled straws and immediately plunged into liquid nitrogen for long-term storage. The warming protocol involves a stepwise dilution of the toxic cryoprotectants and reintroduction of the cells into culturing medium.

All protocols involving cell culture work should be performed in a biological safety cabinet under sterile conditions using aseptic techniques and preferably disposable consumables.

3.4.1 Vitrification of Putative hESCs

1. Prepare correct number of labels for outer straws to be used for vitrification, indicating cell line, passage number, straw number, date and initials of the operator. For the OPS straws, with a black marker write the cell line and passage number on the side and draw a line around the top of each straw (this allows for sight of the straw when encased in the outer straw).

2. Prepare one vitrification 4-well plate to vitrify up to 8 straws (containing 4–6 hESC colony fragments each) from a single cell line.

   (a) To well #1 and well #2 prepare "Bench medium" (20 % FCS in DMEM-HEPES) by addition of 800 μL DMEM-HEPES and 200 μL FCS.

   (b) To well #3 prepare Vitrification Solution 1 (10 % DMSO and 10 % ethylene glycol in Bench medium) by addition of 600 μL DMEM-HEPES, 200 μL FCS, 100 μL DMSO, and 100 μL ethylene glycol.

   (c) To well #4 prepare Vitrification Solution 2 (0.5 M sucrose, 20 % DMSO, and 20 % ethylene glycol in Bench medium) by addition of 50 μL DMEM-HEPES, 250 μL 2 M sucrose solution, 200 μL FCS, 200 μL DMSO, and 200 μL ethylene glycol.

3. Let the vitrification plate warm on a 37 °C warm stage or in the incubator for at least 10 min.

4. Using an ultra-sharp splitting blade, cut colonies of hESCs to be vitrified into small fragments approximately 1 mm × 1 mm.

5. Transfer hESC fragments to wells #1 and/or #2 and allow them to equilibrate in Bench medium for 1 min.

6. Position the liquid nitrogen container, forceps, straws, and putty in the biological safety cabinet so that they are within easy reach. Place the goblet(s) in the cane(s), label the cane tag(s) appropriately, and immerse in the liquid nitrogen.

7. Pipette 20 μL of Vitrification Solution 2 from well #4 to the upturned lid of the plate to make a drop.

8. Using a new tip and avoiding air bubbles, transfer 4–6 fragments in no more than 7 μL from wells #1 or #2 to Vitrification Solution 1 in well #3. Expel the fragments and pipette them a few times. Leave for 25 s.

9. Transfer the fragments from well #3 to the 20 μL drop of Vitrification Solution 2 in a minimum volume and mix thoroughly.

10. Transfer the fragments in approximately 1–2 μL volume to a clean area adjacent to the 20 μL drop, thus making a smaller drop containing all the fragments.

11. Place the narrow end of an OPS straw into the smaller drop to allow capillary action of the straw to aspirate the fragments inside the straw.

12. Plunge the narrow end of the straw into liquid nitrogen. The total time the fragments spend in Vitrification Solution 2 prior to plunging should not exceed 25 s.

13. Using forceps and working in the nitrogen vapor, transfer the OPS straw inside the labeled outer straw. Seal the end of the outer straw with putty then place the straw in the assigned goblet and cane. Avoid getting liquid nitrogen inside the outer straw.

14. Repeat until all hESC fragments have been vitrified.

15. Transfer to liquid nitrogen dewar for long-term storage. Until the hESC fragments are thawed, they should not be subjected to temperature fluctuations as these can cause them to de-vitrify and experience chilling injuries.

3.4.2 Warming of Vitrified Putative hESCs

1. At least one day prior to warming of vitrified hESCs prepare mitotically inactivated fibroblast feeder dish(es) as described in Section 3.2.6.

2. Replace the feeder medium in the feeder dish(es) with warm gas-equilibrated hESC medium and return to incubator until required.

3. Prepare one warming 4-well plate for up to 8 straws from a single cell line.

(a) To well #1 prepare Warming Solution 1 (0.2 M sucrose in Bench medium) by addition of 100 μL 2 M sucrose solution, 700 μL DMEM-HEPES, and 200 μL FCS.

(b) To well #2 prepare Warming Solution 2 (0.1 M sucrose in Bench medium) by addition of 50 μL 2 M sucrose solution, 750 μL DMEM-HEPES, and 200 μL FCS.

(c) To well #3 and #4 prepare Bench medium by addition of 800 μL DMEM-HEPES and 200 μL FCS.

4. Let the plate warm on a 37 °C warm stage or in the incubator for at least 20 min.

5. Transfer the straws to be warmed from the liquid nitrogen dewar into a goblet held in a container with liquid nitrogen and place the container in the biological safety cabinet.

6. Focus the microscope view on the bottom of well #1 of the warming plate.

7. Remove one straw from the goblet with forceps and while working in the liquid nitrogen vapor, cut off the end of the straw containing the putty with scissors, being careful not to cut the OPS straw inside (which can be located by looking for the black ring on the top of the OPS straw).

8. Remove the OPS straw from the outer straw with fine-nosed forceps and let it warm in air for 1–3 s before submerging the narrow end of the straw into well #1 (*see* **Note 28**).

9. Once submerged, immediately cover the top of the straw with a finger. This prevents capillary action and allows the solution containing hESC fragments to drift out of the straw. Observe under microscope to make sure all fragments are expelled.

10. After 1 min transfer the fragments to well #2.

11. After 5 min transfer the fragments to well #3.

12. After 5 min transfer the fragments to well #4.

13. After 5 min transfer the fragments into the prepared dish with feeder cells and carefully place in incubator.

14. Change the hESC medium every second day until ready for further passage, typically 7–10 days after warming.

4   Notes

1. Embryology products developed by Genea are sold through Cook IVF under the company's previous name, Sydney IVF.

2. Mechanical collapsing of embryos is not required.

3. Upon removal of excess vitrification solution, there will be a slight halo effect around the embryo. Hatching blastocysts

and fully hatched blastocysts require less solution to be removed to avoid fixing of the hatched cells to the device.

4. Once vitrified the specimens should not be subjected to temperature fluctuations as they can cause the specimens to de-vitrify and experience chilling injury.

5. Two embryos may be thawed simultaneously; however they should be staggered 5 min apart.

6. Suitable human tissue samples include foreskin (neonatal and adult), skin (with subcutaneous layer attached), aborted fetus, and placental tissue.

7. Whether PBS contains calcium and magnesium is not important; however for convenience only PBS(−) is used as it is required subsequently for cell passaging.

8. The more pieces of tissue that can be plated down at this stage, the less population doublings required to obtain cell numbers sufficient for master stocks. As primary feeder cell lines will senescence after 50 or more passages, and it is possible that early passages may be more supportive of hESC culture, it is better to use the cells at earlier population doublings.

9. Allowing pieces of tissue to partially dry promotes their attachment to the bottom of the flask and improves the outgrowth of fibroblast cells.

10. The number of flasks an operator can successfully passage simultaneously depends on the skill and speed of the operator and the size of the culture flasks (larger flasks take more time to process). Ideally the cells should not be left to dry in between solution changes (no more than 1–2 min) or left to incubate too long in trypsin solution (no more than 6–8 min).

11. The majority of cells (90 % or greater) should be viable after passaging (or thawing), thus negating the need for media change. However, if cultures are initiated from very low cell concentrations and require extended culturing (beyond 7 days), it is advisable to change the medium every 5–6 days.

12. It is important to minimize the time in which cells are resuspended in cold cryoprotectant solution (containing 10 % DMSO) as prolonged exposure can be harmful. As with passaging, the number of flasks an operator can comfortably freeze at one time depends on the skill and speed of the operator and the size of the culture flasks. A general rule of thumb for a single operator is to process no more than four T75 flasks simultaneously. This number can be increased if more than one operator is involved and staggering can be performed to make the overall procedure faster.

13. Labeling can be performed by writing directly onto cryovials with a permanent marker or by using a dedicated labeler and tape compatible with liquid nitrogen storage.

14. For example, if there are 25×10^6 cells in 4 mL of cell suspension and you want to freeze as 0.5×10^6 cells in 0.5 mL aliquots, the final cell concentration should be 1×10^6 cells/mL and thus the final volume of cell suspension to be frozen is 25 mL. As the cells are currently resuspended in 4 mL medium and 2.5 mL (10 %) of the final volume is reserved for DMSO, 18.5 mL of the feeder medium should be added to the cells.

15. Cells should be removed as quickly as possible from cryoprotectant solution as DMSO is harmful to cells; ideally this would occur within 1–2 min of thawing. This limits the number of vials that can be thawed simultaneously, usually being 4 or less vials for a single experienced operator.

16. Assessment of gamma irradiated fibroblast cells for the presence of mitotically active cells can be performed by simply plating feeders at low concentrations and observation for cell proliferation or more accurately by staining for markers of cell proliferation (e.g., Ki-67) and evaluation by high content analysis.

17. This is to compensate for evaporation from dishes and plates with a limited volume of media.

18. The optimal density of mitotically inactivated feeder cells is cell line dependent. We routinely use a higher cell density (6.8×10^4/cm^2) for the derivation of new hESC lines and a lower cell density (2.4×10^4/cm^2) for subsequent expansion and maintenance of established hESC lines.

19. Gas equilibration can be achieved by leaving an appropriate media aliquot in a container with the lid slightly ajar in the incubator overnight. Using gas equilibrated media eliminates pH fluctuation in the culture and although there is no definitive evidence indicating a critical effect on derivation success, it is well known that embryos benefit from steady pH levels.

20. If the embryo is clearly dead, as indicated by the loss of cellular membrane integrity of all blastomeres accompanied with the complete collapsing of a blastocoele and dark appearance of all cells, it may not be worth plating. However, please note that the criteria for embryo viability are quite different from clinical use as the presence of only a few viable ICM cells may be sufficient for hESC derivation.

21. For bisection, the ICM has to be clearly visible and hence only blastocysts with a clear blastocoele cavity and identifiable ICM are suitable. Embryos that have collapsed can be cultured for 1–2 h in blastocyst medium to allow for re-expansion, or can be plated whole for hESC derivation.

22. The zona pellucida can also be removed by incubation with 4 mg/mL Pronase solution as described previously (5), although it is our opinion that mechanical release is the superior method.

23. Avoid transferring oil droplets from the oil covering the culture drop to the Petri dish as they can interfere with visualization.

24. After embryo bisection only the half containing the ICM is transferred to the feeder dish. However, if in any doubt about which half this is, both parts can be plated in the same dish at a distance from each other.

25. Gas atmosphere of 5–6 % CO_2, 5 % O_2, and 89–90 % N_2 are recommended, being the same as for optimal embryo culture.

26. ICM and TE-derived cells can be distinguished as ICM-derived cells appear smaller and tightly packed whereas TE-derived cells appear flatter, larger, and more spread out.

27. The optimal time for first passage will vary depending on if the embryo was plated whole or after bisection, and if bisected, how much TE-derived cells were plated together with the ICM. Ideally the bud is easily distinguished, compact, and consisting of tens or hundreds of cells. If the bud is tightly surrounded by TE-derived cells, it should be passaged as early as 4 days after plating. However, if the bud is not completely surrounded by TE-derived cells, it can be left to grow for up to 10 days or longer, although here the age of the feeder plates will be the limiting factor as they should be no older than 10–14 days (after feeder cell plating).

28. Handle the straw carefully and especially avoid the liquid nitrogen shooting out of the wider end of the straw once it is lifted out of the liquid nitrogen. It is highly recommended to wear safety goggles.

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Characterizing Pluripotent Stem Cells Using the TaqMan® hPSC Scorecard™ Panel

Jeffrey Fergus, Rene Quintanilla, and Uma Lakshmipathy

Abstract

Rapid technological developments for the efficient generation of footprint-free induced pluripotent stem cells (iPSC) enabled the creation of patient-specific iPSC for downstream applications in drug discovery and regenerative medicine. However, the large number of iPSCs, generated from diverse genetic backgrounds using various methods and culture conditions, created a steep challenge for rapid characterization and a demand for standardized methods. Current methods rely on a combination of in vitro and in vivo cellular analyses based on the expression of markers of self-renewal and the ability of the cells to differentiate into cell types representative of the three germ layers as a confirmation of functional pluripotency. These methods, though informative and extensively used, are not ideal for parallel analyses of large numbers of samples and hence not amenable to high-throughput environments. Recently, genetic and epigenetic expression signatures were used to define and confirm cell states, thus providing a surrogate molecular assay that can potentially replace complex in vivo cellular assays such as teratoma formation.

In this chapter, we describe a molecular assay for rapid characterization and standardization of pluripotent stem cells. The TaqMan® hPSC Scorecard™ Panel is a comprehensive gene expression real-time PCR assay that consists of 94 individual q-PCR assays comprised of a combination of control, housekeeping, self-renewal, and lineage-specific genes. The resulting expression data set is analyzed using cloud-based analysis software that compares the expression pattern against a reference standard composed of multiple functionally validated ESC and iPSC lines. This system was successfully used to test several ESC and iPSC lines in their undifferentiated states to confirm their signatures of self renewal, as well as their terminally differentiates states, via spontaneous differentiation and directed differentiation into specific lineages, to determine the lines' differentiation potential. This genetic analysis tool, together with the flexibility to utilize varying sample inputs and preparation methods, provides a rapid method to confirm functional pluripotency of ESCs and iPSCs.

Keywords: Pluripotent stem cells, Molecular characterization, TaqMan® hPSC Scorecard™ assay, Gene expression assay, Trilineage differentiation potential, Self-renewal

1 Introduction

The ability to generate footprint-free iPSC using reprogramming technologies led to the generation of patient-derived stem cells from various tissue sources and patients with various genetic backgrounds and disease states. In addition, a variety of methods and media systems were created for reprogramming (1, 2). This wide availability of iPSCs derived from various sources and by various

methods is revolutionizing the field of regenerative medicine. iPSCs promise to be valuable tools in studying complex diseases, cell-based drug screening, and personalized medicine (3, 4). The bottleneck in this process shifted from efficient methods of iPSC generation, to a lack of high-throughput and cost-effective tools for characterization and standardization.

Human pluripotent stem cells (hPSCs) are traditionally monitored based on morphology and characterized using a panel of tests assessing differential marker expression to test self-renewal and differentiation potential (5). The ability of iPSC to form teratomas in vivo is often considered the most stringent assay for human stem cells and is based on histological and immunohistochemical analysis of tumors formed in mice injected with PSCs to confirm the presence of all three germ layers (6, 7). Although definitive, this method requires 1–2 months to complete, is difficult to standardize, is expensive, and highly variable (8). There is therefore a need for a cost-effective, animal-free alternative for assessing functional pluripotency (9).

To overcome these constraints, molecular methods that are simple, accurate, animal-free, and cost-effective are being developed. One such test called "PluriTest™" is a robust open-access bioinformatics assay that compares gene expression against a database consisting of transcriptional profiles of normal human ESC, iPSC, and a variety of differentiated cell types and adult human tissue (10). However, studies report that there are similarities (11) and differences (12) between human ESC and iPSC (13) in general. Such subtle differences between the various pluripotent lines could potentially manifest as random variation in differentiation propensity (14–16). A second molecular assay called the "Scorecard" is based on expression profiling of lineage-specific genes in differentiating embryoid body and comparison to reference transcriptome maps generated using several ESC and iPSC lines (17). The Taq-Man® hPSC Scorecard™ Panel was based on this concept and developed in collaboration with Alex Meissner. The assay comprises 94 predefined markers for self-renewal, Ectoderm, Mesoderm, and Endoderm and cloud-based analysis software that compares the expression pattern to a functionally validated reference standard generated with a combination of ESC and iPSC lines. This method is simple, easy, and flexible to allow analysis of cells cultured under varying conditions and RNA and cDNA prepared using different methods. Both undifferentiating and cells spontaneously differentiating via embryoid body formation are analyzed to confirm the expression of self-renewal markers and trilineage differentiation markers, thus confirming functional pluripotency.

In this chapter we describe methods for sample generation, isolation of RNA for the preparation of cDNA and Scorecard™ analysis for functional pluripotency confirmation of cells cultured on feeders and feeder-free in different media conditions.

2   Materials

2.1   Reagents

1. DMEM/F-12 (1×), Liquid (1:1), with GlutaMAX™-I, *Life Technologies Cat #: 10565-018.*

2. Essential 8™ Medium, *Life Technologies Cat #: A1517001.*

3. StemPro® hESC SFM, *Life Technologies Cat #: A1000701.*

4. MEM Non-Essential Amino Acids Solution (100×), *Life Technologies Cat #: 11140-050.*

5. KnockOut™ Serum Replacement, KSR, *Life Technologies Cat #: 10828-028.*

6. 2-Mercaptoethanol, *Life Technologies Cat #: 21985-023.*

7. Geltrex® LDEV-Free hESC-Qualified Reduced Growth Factor Basement Membrane Matrix, *Life Technologies Cat #: A1413301.*

8. Collagenase, Type IV, powder, *Life Technologies Cat #: 17104-019.*

9. StemPro® EZPassage™ Disposable Stem Cell Passaging Tool, *Life Technologies Cat #: 23181-010.*

10. BD Falcon Cell Scraper, *Fisher Scientific Cat #: 08-771-1A.*

11. FGF-basic (AA 1-155) Recombinant Human, *Life Technologies Cat #: PHG0264.*

12. D-PBS without $CaCl_2$ and $MgCl_2$ (1×), Liquid, *Life Technologies Cat #: 14190-250.*

13. TRIzol®, *Life Technologies Cat # 5596-026.*

14. Chloroform, *Sigma Cat #: C-2432.*

15. Isopropanol, *Sigma Cat #: I9516-500 mL.*

16. Ethanol, *Sigma Cat #: E7023-500 mL.*

17. RNase-free Water, *Life Technologies Cat #: 10977.*

18. DNA-*free*™ Kit, *Life Technologies Cat #: AM1906.*

19. High-capacity cDNA Reverse Transcription kit with RNase Inhibitor, 200 reactions, *Life Technologies Cat #: 4374966.*

2.2   Media

1. PSC Medium (For 100 mL): 79 mL DMEM/F-12, 20 mL KSR, 1 mL NEAA, 100 μL 2-Mercaptoethanol.

   (a) Medium lasts for up to 28 days at 4 °C. Add bFGF (final concentration 4 ng/mL) fresh prior to use (example 0.4 μL reconstituted bFGF per mL of medium).

2. EB Medium (For 100 mL): 79 mL DMEM/F-12, 20 mL KSR, 1 mL NEAA, 100 μL 2-Mercaptoethanol,

   (a) Medium lasts for up to 28 days at 4 °C.

3. Basic FGF Solution (10 μg/mL, for 1 mL): 10 μg Basic FGF, 990 μL D-PBS, 10 μL KSR.

   (a) Aliquot and store at −20 °C for up to 3 months. Once bFGF aliquot is thawed, use within 7 days, when stored at 4 °C.

4. Collagenase IV Solution (1 mg/mL, For 50 mL): 50 mg Collagenase IV, 50 mL DMEM/F-12.

   (a) Sterilize through 0.22 μm filter and store at 4 °C for up to 14 days.

5. Cell Culture.

   (a) Cells cultured under feeder-free conditions using matrices and feeder-free media or cells on feeders adapted to feeder-free conditions using Geltrex® and MEF conditioned media for at least one passage.

2.3 Scorecard Reagents

- TaqMan® hPSC Scorecard™ Panel, 384-well, *Life Technologies Cat #: A15870.*
- TaqMan® hPSC Scorecard™ Kit, 384-well, *Life Technologies Cat #: A15872.*
- TaqMan® hPSC Scorecard™ Panel, Fast 96-well, *Life Technologies Cat #: A15876.*
- TaqMan® hPSC Scorecard™ Kit, Fast 96-well, *Life Technologies Cat #: A15871.*
- MicroAmp® Optical Adhesive Film, *Life Technologies Cat #: 4311971.*
- TaqMan® Gene Expression Master Mix, *Life Technologies Cat #: 4369016®.*
- TaqMan® Fast Advanced Master Mix, *Life Technologies Cat #: 4444558.*
- Pipettes, multichannel pipettes, plates, tubes, etc.

3   Supported Instruments

- QuantStudio™ 12 K Flex.
- ViiA™ 7.
- StepOnePlus™ Real-Time PCR System.
- QuantStudio™ 6/7 Flex Real-Time PCR system.
- 7500 Fast Real-Time PCR System.
- 7900HT Fast Real-Time PCR System.

4   Methods

4.1   Sample Generation

4.1.1   Generation of Undifferentiated Cells

1. For cells cultured on Murine embryonic fibroblast (MEF) feeders, cells must be cultured under feeder-free condition on Geltrex®-matrix-coated culture vessels in MEF-conditioned medium for at least one passage (Note 1). For feeder-free cultures, you may harvest directly from the culture dish.

2. Prior to harvesting for RNA isolation, cells need to be observed under phase contrast microscope to confirm homogeneous morphology with little or no differentiation (Note 2).

3. Remove growth media from cells and wash once with D-PBS.

4. Add 0.5–1 mL of TRIzol® directly on the cells and gently swirl until the liquid is equally distributed across the plate and incubate at room temperature for 3–5 min (Note 3).

5. Use a sterile cell scraper to collect the TRIzol®-treated cell slurry.

6. Using a 1-mL pipette, carefully remove all the lysed cells into a sterile DNase/RNase-free tube. Store it at -80 °C until ready for RNA isolation.

4.1.2   Differentiated Cells

Suspension Embryoid Bodies (EBs) from Feeder-dependent PSC cultures

1. Culture PSC on iMEF until 80–90 % confluence in culture dishes (Note 3).

2. Aspirate the culture medium from plates or dishes. Add 1 mL prewarmed PSC Medium to each well of 6-well plate, 2 mL to each 60-mm dish or 6 mL to each 100-mm dish.

3. Roll the StemPro® EZPassage™ disposable stem cell passaging tool across the entire dish or plate in one direction (left to right). Rotate the culture dish or plate 90°, and roll the StemPro® EZPassage™ disposable stem cell passaging tool across the entire dish or plate.

4. Use a cell scraper to gently detach the cells off the surface of the plates or dishes.

5. Gently transfer cell clumps using a 5-mL pipette and place into a 15-mL conical tube (Note 4).

6. Add 1 mL prewarmed PSC Medium to each well of 6-well plate, 2 mL to each 60-mm dish or 3 mL to each 100-mm dish to collect residual cells and add cell suspension to the tube.

7. Allow the cell clumps to gravity sediment in the hood for 5–10 min and aspirate off the supernatant that may contain single cells and possible MEF contaminants.

Table 1
Medium volume recommendations for EB culture

Culture vessel	Surface area (cm^2)	Volume of medium required (mL)
6-well plate	10 cm^2/well	2 mL/well
12-well plate	4 cm^2/well	1 mL/well
24-well plate	2 cm^2/well	0.5 mL/well
35-mm dish	10 cm^2	2 mL
60-mm dish	20 cm^2	5 mL
100-mm dish	60 cm^2	10 mL

8. Transfer the cell clumps to a 60-mm Petri (non TC) dish in a total of 5 mL of PSC medium. Generally use one plate of PSC per one plate of EBs to be formed, in total volumes listed in Table 1.

9. Place the dishes containing the cell clumps in a 37 °C incubator.

10. After 24 h, remove the PSC medium from the Petri dish by transferring the contents of the dish to a 15-mL conical tube and allowing the cell clumps to sediment down via gravity in the hood for 5–10 min.

11. Aspirate off the spent PSC Medium and replace with 5 mL of fresh EB Medium and transfer the cell clumps to a fresh 60-mm Petri dish. Place in the incubator.

12. Add fresh EB Medium every other day for a minimum of 4 days. Use the same procedure of sedimenting the EBs.

13. Continue EBs in suspension for 5–7 days and then harvest cells for Scorecard^{TM} analysis.

Suspension Embroid Bodies (EBs) from Feeder-Free PSC cultures

1. Culture PSC on Geltrex™-coated dishes in feeder-free media such as MEF conditioned media, Essential 8™ or Stem Pro® hESC SFM until 80–90 % confluence.

2. Aspirate medium from culture vessel, wash culture vessel once with DPBS ($-/-$) and aspirate.

3. Add an appropriate volume of Collagenase IV Solution to culture vessel. Ensure complete coverage of culture surface with Collagenase IV Solution (Note 5).

4. Incubate at 37 °C until cells detach; time varies but typical incubation times range from 30 to 60 min. Gently tap culture vessel to determine if the cells have detached.

5. Add enough PSC culture medium supplemented with 4 ng/μL bFGF to dilute the Collagenase IV solution 1:4 and wash cells off the culture vessel (Note 6).

6. Transfer cell clumps to a 15 or 50-mL conical and gravity sediment for 5–10 min.

7. Discard supernatant and resuspend the cell pellet with 5 mL of PSC medium supplemented with 4 ng/mL bFGF.

8. Transfer all 5 mL of cells to a 60-mm non-TC-treated dish and culture overnight at 37 °C.

9. After 24 h, remove PSC medium from the Petri dish by transferring the contents of the dish to a 15-mL conical tube and allowing the cell clumps to sediment down via gravity in the hood for 5–10 min.

10. Aspirate off the spent PSC Medium and replace with 5 mL of fresh EB Medium and transfer the cell clumps to a fresh 60 mm Petri dish. Place in the incubator.

11. Add fresh EB Medium every other day for a minimum of 4 days. Use the same procedure of sedimenting the EBs.

12. Continue EBs in suspension for 5–7 days and then harvest cells for Scorecard™ analysis.

Harvesting EBs in TRIzol® Reagent

1. On day 5–7 of EB suspension, gently transfer the cells and the medium from the Petri dish into a 15-mL conical tube. Use an additional 5 mL of DPBS $(-/-)$ to collect any remaining EBs from the culture dish and add into the conical tube.

2. Allow the EBs to sediment down by gravity for 10–15 min, and then aspirate off the supernatant (i.e., spent EB medium).

3. Using a P1000 pipettor, add 1 mL of TRIzol® reagent and pipette up and down to assist in properly breaking up the cell clumps.

4. Incubate the EBs for 3–5 min. Repeat pipetting and incubation, if the EBs requires more time to be lysed. Collect the slurry into a sterile RNAse-free microcentrifuge tube. Store at −80 °C until ready for RNA isolation.

4.2 Sample Preparation

4.2.1 Total RNA Preparation

1. Incubate the lysate with TRIzol® Reagent at room temperature for 5 min to allow complete dissociation of nucleoprotein complexes.

2. To the TRIzol® lysate add 0.2 mL Chloroform per 1 mL of TRIzol® reagent and shake the tube vigorously for 15 s.

3. Incubate at room temperature for 2–3 min and centrifuge at $12,000 \times g$ for 15 min at 4 °C.

4. Carefully remove the upper aqueous phase and transfer to a new tube.

5. Add 0.5 mL 100 % isopropanol to the aqueous phase per 1 mL of TRIzol® reagent; incubate at room temperature for 10 min.

6. Centrifuge at $12,000 \times g$ for 10 min at 4 °C.

7. Carefully remove the supernatant from the RNA pellet and wash with 1 mL of 75 % ethanol.

8. Centrifuge the tube at 7,500 × g for 5 min at 4 °C. Discard the supernatant and air-dry the RNA pellet for 5–10 min.

9. Resuspend the RNA pellet with 20–50 µL RNase-free water.

4.2.2 DNase Treatment

1. Thaw DNA-*free*™ Kit reagents on ice.

2. Add 0.1 times the RNA volume of 10X DNase I Buffer and 1 µL rDNase I to the RNA in a clean DNase/RNase-free eppendorf tube, and mix gently (Note 7).

(a) RNA sample	1–10 µg
(b) 10× DNase I reaction buffer	5 µL
(c) rDNase I (2 U)	1 µL
(d) DEPC-treated water to bring reaction to 50 µL	X µL
Total	*50 µL*

3. Incubate the tube at 37 °C for 20–30 min.

4. Add the resuspended DNase Inactivation Reagent (typically 0.1 times the RNA volume) and mix well.

5. Incubate 2 min at room temperature, mixing occasionally.

6. Centrifuge at 10,000 × g for 1.5 min and transfer the RNA to a fresh tube.

4.2.3 RNA Quantification

1. Use NanoDrop to quantify the extracted RNA sample. Quality of RNA is best assessed using Absorbance 260/280 ratio, with the recommended value close to 2.0.

2. RNA integrity can be further assessed by running the samples on a 1 % Agarose gel and assessing the 2:1 ratio of the 28 and 18 s RNA bands and the absence of degraded RNA that appears as a small molecular weight smear.

3. If using a Bioanalyzer, a RIN (RNA integrity number) value of higher than 5 may be sufficient, but higher than 8 is ideal for downstream applications.

4.2.4 Generate cDNA by Reverse Transcription

1. Allow the components of the High-capacity cDNA Reverse Transcription Kit with RNase Inhibitor to thaw on ice.

2. Prepare a 2× RT master mix by mixing the following components as listed in Table 2.

3. Place the 2× RT master mix on ice and mix gently.

4. Prepare the RNA samples by diluting 1 µg total RNA in a total of 225 µL of RNase-free water.

Table 2
Volumes for 2× RT master mix

2× Master Mix	×1	×10 (1 Sample)	×38 (1 × 384 w or 4 × 96 w plates)
10× RT Buffer	5	50	190
25× dNTP Mix	2	20	76
10× RT Primers	5	50	190
MultiScribe™ RT	2.5	25	95
RNase Inhibitor	2.5	25	95
Nuclease-free Water	8	80	304
Total	25 μL	250 μL	950 μL

5. Add 225 μL of 2× RT master mix to the diluted RNA and mix well.

6. Aliquot 50 μL of the above RNA plus RT mix in eight vertical wells of a 96-well plate or an eight-strip PCR tube (Fig. 1).

7. Run the RT reaction in a thermal cycler using conditions as listed in Table 3.

8. Proceed to TaqMan® qRT-PCR, Section 4.2.5. If you do not proceed immediately to PCR amplification, store all cDNA samples at −15 to −25 °C. To minimize freeze–thaw cycles, store the cDNA in smaller aliquots.

4.2.5 Perform TaqMan® qRT-PCR

1. Dilute each well containing 50 μL cDNA with 20 μL PCR water for a final volume of 70 μL.

2. Add 70 μL 2× TaqMan® Gene Expression Master Mix (if using the TaqMan® hPSC Scorecard™ Panel 384 w) or 70 μL 2× TaqMan® Fast Advanced Master Mix (if using the TaqMan® hPSC Scorecard™ Panel 96 w FAST). Spin down the plates at 600 × g for 2 min.

3. Load 10 μL per well using multichannel pipette onto the 384-well or the 96-well plate using fresh tips each time as shown below. For 96-well plates, one well is sufficient to load one row of the plate (Fig. 2).

4. Seal the plate with the MicroAmp® Optical Adhesive Film, and centrifuge it at 600 × g for 2 min.

5. Place the plate in a compatible RT-PCR instrument equipped with the appropriate thermal block (Note 8).
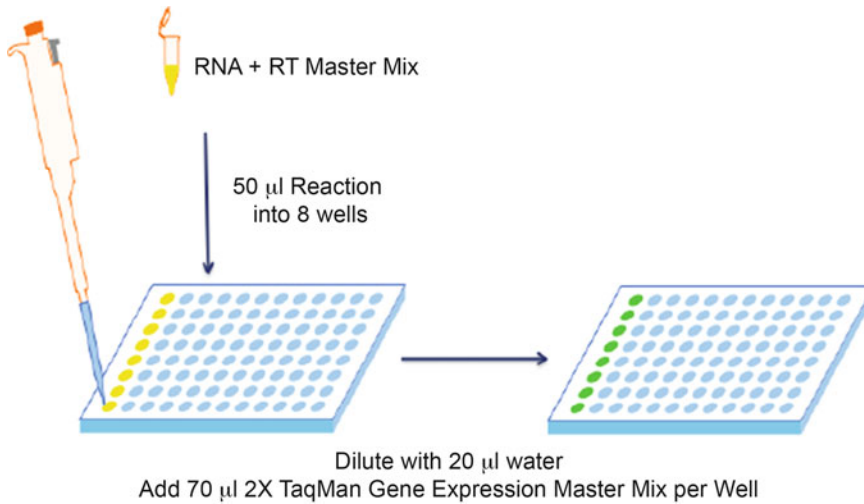
Fig. 1 Sample setup for cDNA synthesis

Table 3
Thermal cycler condition for RT reaction

Step	Temperature (°C)	Time
1	25	10 min
2	37	120 min
3	85	5 min
4	4	Hold

6. Open the experiment template file (.edt) and save a separate copy (.eds) with your experimental details. Run the experiment using Standard method for 384-well plates with the TaqMan® Gene Expression Master Mix and Fast mode for 96-well plates with the TaqMan® Fast Advanced Master Mix, using the cycling parameters already in the experiment template file (.edt) (Note 9).

4.2.6 Analyze Data Using the hPSC Scorecard™ Analysis Software

1. Analyze the gene expression data from the TaqMan® hPSC Scorecard™ Panels using the web-based hPSC Scorecard™ Analysis Software, available at www.lifetechnologies.com/scorecarddata.

2. The hPSC Scorecard™ Analysis Software summarizes all key experimental results, including pluripotency and differentiation potential on a single dashboard. It also allows you to tag and filter experiments, view expression, correlation, box plots, heat maps, scores, and export experimental results and data as a PDF or as a spreadsheet.
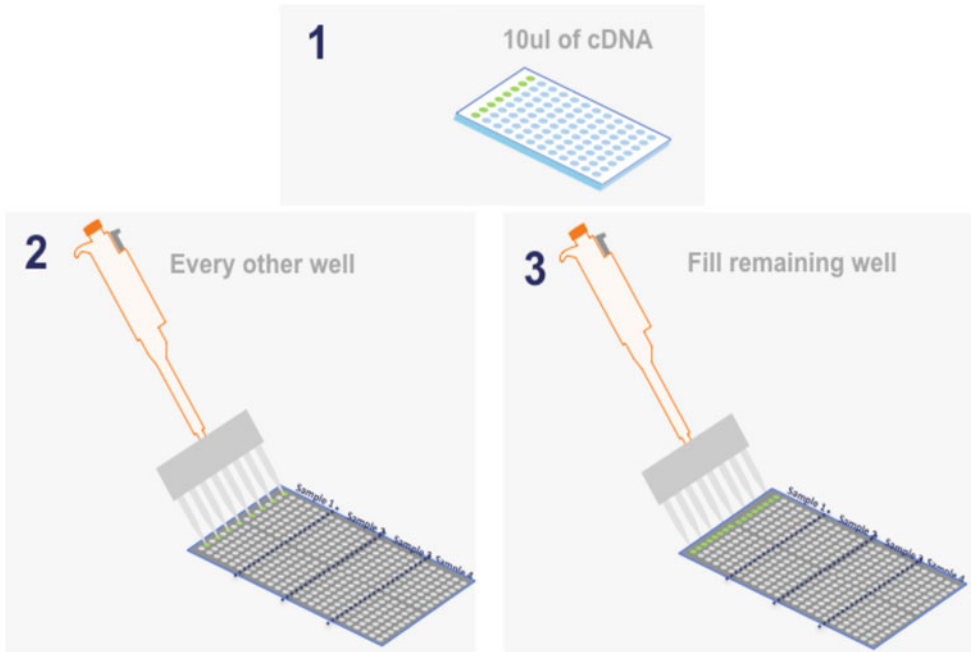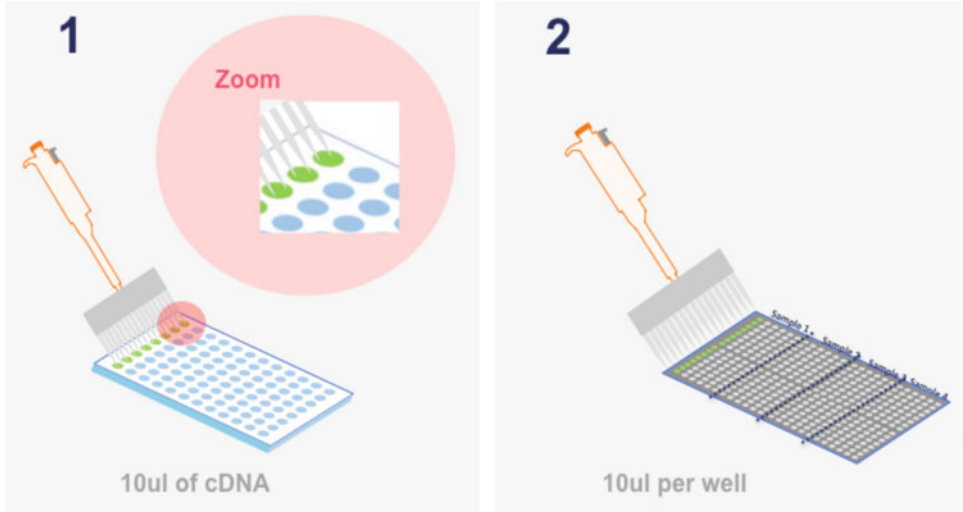
a Loading 384-well plates with 8-Channel Pipette

b Loading 384-well plates with 16-Channel Pipette

Fig. 2 Transfer of diluted cDNA to 384w or 96w TaqMan® hPSC Scorecard™ plates

5   Notes

1. The majority of the primers in the TaqMan® hPSC Scorecard™ panel are human specific but a few primers that cross-react with mouse can impact results. If cultured on human feeders, extra precaution is required to completely eliminate residual presence of human feeders since it can contribute to gene expression and interfere with the outcome of results.

2. Greater than 95 % of the cells should be expressing markers of self-renewal, such as the surface marker SSEA4. New users are strongly suggested to correlate their visual observation with marker expression to confirm quality of cells used for gene expression analysis.

3. This protocol is not for PSCs cultured in flasks because Stem-Pro® EZPassage™ disposable stem cell passaging tool and cell scraper cannot reach the cells in flask. If PSCs are grown in flasks, it is recommended that the colonies be harvested using Collagenase IV, as if normally passaging cultures, collect the colonies and triturate to make small cell clumps and proceed to step 8 in the following protocol.

4. Do not break cell clumps into small pieces.

5. Collagenase IV Solution needs to be prewarmed to 37 °C.

6. Be very gentle and do not triturate too many times. Large clumps are critical for successful EB formation in feeder-free systems such as Essential 8™.

7. For routine DNase treatment (remove 2 μg of genomic DNA from 50 μL reaction with ≤200 μg/mL nucleic acid).

8. TaqMan® hPSC Scorecard™ Panel 96w FAST must be run on RT-PCR systems that contain Fast thermal cycling blocks and the TaqMan® hPSC Scorecard™ Panel 384w must be run on systems with standard thermal cycling blocks.

9. The experiment template files (.edt) are available at www.lifetechnologies.com/scorecardinstrument. Refer to the appropriate instrument user guide for information on how to set up the plate document/experiment or create a template from the setup file.

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Growth of Human Pluripotent Stem Cells Using Functional Human Extracellular Matrix

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Abstract

The use of animal products in the derivation and maintenance of human pluripotent stem cells (hPSCs) limits their possible applications in research and in clinics. Thus, one of the major goals in regenerative medicine is the establishment of animal-free conditions to support the culture and differentiation of human stem cells. Human fibroblasts produce an extracellular matrix (ECM) which can be extracted without the use of detergents, sterilized, and then used to coat tissue culture plates.

We have shown that human embryonic stem cells (hESCs) grown on this matrix maintain their pluripotency in the presence of medium conditioned by fibroblast cells, and that these cells maintain expression of surface proteins (SSEA4, Tra1-60, Tra1-81), alkaline phosphatase activity, and specific intracellular markers (Nanog, Oct-4, Tert, FoxD3) in hESCs. This growth system reduces exposure of hPSCs to feeder layers and animal ingredients, thereby limiting the risk of pathogenic contamination and additionally, facilitating their manipulation. Herein we present an improved version of our previous protocol for extracting ECM from human foreskin fibroblast using a different buffer. Our new hypotonic shock method is detergent-free, reduces costs, and preserves the integrity of the extracted ECM. This improved protocol has been validated for undifferentiated-state hPSC maintenance (more than 40 passages), stem cell differentiation, and for cell migration assays.

Keywords: Human embryonic stem cells, Human pluripotent stem cells, In vitro growth, Extracellular matrix, Hypo-osmotic lysis buffer

1 Introduction

The stem cell biology field is continually transforming, especially in recent years with the introduction of several validated protocols for obtaining human-induced pluripotent stem cells (hiPSCs) ([1], [2]), which have created new avenues for studying different diseases and regenerative medicine applications. Several human embryonic stem cell (hESC) and hiPSC lines have been derived and grown using mouse feeder layers, Matrigel®, or other animal reagents ([3], [4]). However, recent successes using pluripotent stem cell (PSC) derivatives in clinical trials ([5], [6]) have emphasized the need for improved quality standards before these derivatives are applied in cell therapies. In particular, new culture methods must be developed that circumvent the need for animal products because

these might provoke infections or immune rejection following transplantation into patients (7). Thus, chemically defined culture systems that are devoid of nonhuman substances will greatly facilitate the use of human stem cells (hSCs) in therapies.

Extracellular matrix (ECM) modulates cellular adhesion processes and signaling inside stem cell niches and is essential for maintaining their structure and supportive qualities (8, 9). An attractive alternative to the use of feeder cells, and the complications associated with them, is the development of an animal-free ECM, and there would be many advantages to maintaining PSCs on such supports. Indeed, undefined mixtures of natural and synthetic matrix proteins are already used as a coating for in vitro hSC culture (10), but unfortunately, most of these reagents are cost-prohibitive for many laboratories. Decellularization procedures are traditionally used to isolate ECM from cells and organs (11), facilitating remodeling in animal models and humans. However, the procedures used to remove the cells, especially detergent-based methods, can alter the native structure of the ECM (12, 13).

We previously published a protocol which allows long-term growth of undifferentiated hESCs on human functional foreskin ECM (hffECM), extracted by using RIPA buffer and cultured using human foreskin conditioned medium, in the *Methods in Molecular Biology* series (14). Herein we present a modification of our previous protocol which replaces RIPA with a simple, low-cost hypotonic buffer without detergents. The hffECM obtained is capable of maintaining the pluripotency of hESCs for more than 40 passages (12). Additionally, our hffECM has also been validated as a coating for directed-differentiation experiments by using both hESCs and mesenchymal stem cells (MSCs) and as a basis for cell migration studies (12, 15, 16).

2 Materials

2.1 Culture of Human Foreskin Fibroblasts (hFFs) for Derivation of Conditioned Medium and Human Functional Foreskin-Derived Extracellular Matrix (hffECM)

1. Human foreskin fibroblasts (hFFs) (ATCC, Catalogue No. CRL-2429, Passages 11–18; *see* **Note 1**).

2. Iscove's medium (Sigma, St. Louis, MO), supplemented with 10 % human serum (HS), 1 % Glutamax (GIBCO, Invitrogen, Carlsbad, CA).

3. Gelatin (1.5 %; Sigma). In a sterile bottle, add embryo-tested water (Sigma) to gelatin. Warm the mixture to 37 °C in a water bath using a shaker. Store at 4 °C or make aliquots and keep them frozen at −20 °C. These aliquots can be stored for up to 6 months. Before proceeding with tissue culture, thaw the aliquots and dilute them to 0.01 % with sterile Dulbecco's phosphate buffer saline (DPBS) (Invitrogen). Pass the solution through a 0.22 μm filter (Nalgene, Hereford, UK). Coat the

culture surfaces by pipetting 1 mL/well into 6-well plates (BD, San Jose, CA) or 12 mL into a 75 cm^2 flask (Iwaki, Ibaraki, Japan). Allow the gelatin to settle at 37 °C for 30 min. Plates may be used immediately or stored at 4 °C to prevent evaporation.

4. Mitomycin C (Fluka, Buchs, Switzerland) is dissolved in Iscove's medium (Sigma) at 1 mg/mL, stored at 4 °C and then added to the cultures as required.

5. Tryple Select (GIBCO, Invitrogen) is used instead of trypsin to detach cells from the tissue culture plates.

6. DPBS without Ca^{2+} or Mg^{2+}, pH 7.4 (GIBCO, Invitrogen).

7. Trypan Blue (Sigma) to count and evaluate cell viability (*see* **Note 2**).

8. Neubauer haemocytometer (Brand, Wertheim, Germany).

2.2 Derivation of Conditioned Medium

As previously described (17), TESR1 medium is usually used as the base medium for hFFs conditioning, using passage 11–18 cells.

1. TERS1 conditioned medium: after collection medium can be frozen at −80 °C for up to 6 months (*see* **Notes 3** and **4**).

2.3 Preparation of Human Functional Foreskin-Derived Extracellular Matrix (hffECM)

1. Osmotic lysis buffer Tris–EDTA (TE) pH 7.4: 10 mM Tris–HCl pH 7.4, 1 mM EDTA pH 8 (all from Sigma). For preparation details *see* **Note 5**.

2. Cell scrapers (Falcon, BD Biosciences, Madrid).

3. DPBS (GIBCO, Invitrogen).

2.4 Preparation of Samples for Transmission Electron Microscopy (TEM)

1. PB (EMS).

2. Glutaraldehyde (3 %): dilute glutaraldehyde (Electron Microscopy Science [EMS], Hattfield, PA) from a 25 % (v/v) stock in PB.

3. Petri dish (Falcon, BD).

4. Disposable Pasteur pipettes (Falcon).

5. Osmium 1 % (Sigma): prepared by diluting osmium in PB.

6. Uranyl acetate (2 %) diluted in 70 % ethanol.

7. Preparation of alcohol gradient: use the appropriate volume of absolute ethanol (Merck), mixed with distilled water to prepare 30 %, 50 %, 70 %, and 90 % ethanol solutions.

8. Resin LR-White (EMS).

See **Note 6** for safety measures that must be adopted to perform this technique.

2.5 Preparation of Samples for Atomic Force Microscopy (AFM)	1. 0.1 M DPBS (Gibco).
	2. 2 % paraformaldehyde/2.5 % glutaraldehyde/PB (all from EMS).
	3. Series of ethanol/water solutions (30 %, 50 %, 70 %, 90 %, 96 %). All from Sigma.
	4. Absolute ethanol (Sigma).

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3. Series of ethanol/water solutions (30 %, 50 %, 70 %, 90 %, 96 %). All from Sigma.

4. Absolute ethanol (Sigma).

2.6 Culture and Maintenance of Undifferentiated hESC Lines

1. H9 and H1 hESC lines (WiCell, Madison, WI).

2. TERS1 conditioned medium.

3. TGF-β1 (Invitrogen): reconstituted with sterile 4 mM HCl (Sigma) containing 1 mg/mL of human serum albumin (Sigma) to a final stock solution concentration of 40 ng/mL. Store in 50 μL aliquots at −20 °C.

4. Human recombinant basic fibroblast growth factor (bFGF; Invitrogen) is dissolved in 1 mL DMEM Knockout Medium (Invitrogen) and stored in 100 μL aliquots at −20 °C.

2.7 Analysis of Undifferentiated hESC Markers

2.7.1 Staining of Pluripotency Cell Surface Markers by Immunocytochemistry

Antibodies that detect specific cell-surface hESC markers are commercially available from Chemicon and antibodies for detection of the ECM component fibronectin are available from Sigma. Secondary antibodies are all commercially available from Invitrogen. *See* Tables 1 and 2 for recommended dilutions and providers.

1. DPBS without Ca^{2+}, Mg^{2+} (GIBCO, Invitrogen).

2. 4 % paraformaldehyde (*see* recipe at Sect. 2.7.2, step 2).

3. 0.05 % sodium azide (Sigma) in DPBS.

4. Triton-X-100 (Sigma): prepare a dilution of 1 % Triton-X-100 in DPBS to permeabilize the hESCs.

5. Blocking solution: 4 % serum in DPBS. Serum for the blocking solution should be of the same origin/animal as the secondary antibody.

6. Prolong gold anti-fade reagent with DAPI (Invitrogen). *See* **Note** 7.

2.7.2 Alkaline Phosphatase (AP) Detection Kit (Chemicon Millipore, Billerica, MA)

1. This kit provides two components for AP detection: Fast Red Violet solution (0.8 g/L stock) and naphthol AS-BI phosphate solution (4 mg/mL) in AMPD buffer (2 mol/L), pH 9.5.

2. Paraformaldehyde 4 % in DPBS: Prepared fresh with distilled water. To prepare 100 mL, heat 50 mL distilled water to 60 °C on a hot plate in a fume hood (do not exceed 65 °C) and add 4 g of paraformaldehyde powder. Stir the solution until it becomes clear (a few drops of NaOH can be added). After

Table 1
List of primary antibodies

Primary antibody	Isotype	Working dilution	Catalogue number	Provider
SSEA4*	IgG3	1:100	MAB 4304	Chemicon
Tra1-81*	IgM	1:100	MAB 4381	Chemicon
Tra1-60*	IgM	1:100	MAB 4360	Chemicon
Fibronectin	IgG1	1:100	F0916	Sigma

*SSEA4, Tra1-81, Tra1-60, and AP can also be acquired as a kit (SCR001; Chemicon)

Table 2
List of secondary antibodies

Secondary antibody	Working dilution	Catalogue number	Provider
Alexa Fluor against IgG	1:500	A11029	Invitrogen
Alexa Fluor against IgM	1:500	A21042	Invitrogen
Alexa Fluor against IgG1	1:500	A21124	Invitrogen

that, filter the solution through a 0.22 μm filter and add 50 mL of sterile DPBS at pH 7.4 (*see* **Note 8**).

3. TBST 1× Rinse Buffer: prepared fresh with 20 mM Tris–HCl, pH 7.4, 0.15 M NaCl, 0.05 % Tween-20 (all from Sigma).

2.7.3 Intracellular Markers by RT-PCR and QPCR Analysis

See Table 3 for the complete list of primers and reaction conditions.

1. RNeasy mini kit (74104, Qiagen).

2. Deoxyribonuclease (DNase) I (RNasa-free DNase SET; 79254; Qiagen).

3. High Capacity cDNA RT kit (PN 4368814; Applied Biosystems).

4. FastStart PCR Master (04710436001; Roche).

5. Power SYBR® Green PCR master mix (4367659; Applied Biosystems).

6. PCR microplates.

7. 1.5 mL sterile microcentrifuge tubes (Eppendorf, Hamburg, Germany).

Table 3
List of primers and conditions used for both RT-PCR and qPCR

Gene name	Primer sequences	Annealing temperature (°C)	Size (bp)	Detection
Housekeeping gene				
ß2-microglobulin	F: CTCGCGCTACTCTCTCTTTCTG	55	335	RT-PCR QPCR
	R: GCTTACATGTCTCGATCCCACT			
Pluripotency assay genes				
Nanog	F: GGTGGCAGAAAAACAACTGG	60	235	RT-PCR
	R: CATCCCTGGTGGTAGGAAGA			
Nanog	F: AATGGTGTGACGCAGAAGG	60	157	QPCR
	R: ACTGGATGTTCTGGGTCTGG			
Oct-4	F: cctgtctccgtcaccactct	60	128	RT-PCR QPCR
	R: CAAAAACCCTGGCACAAACT			
FoxD3	F: CAGAGCCCGCAGAAGAAG	60	133	RT-PCR
	R: CGAAGCAGTCGTTGAGTGAG			
FoxD3	F: GCCCAAGAACAGCCTAGTGA	60	254	QPCR
	R: GGGTCCAGGGTCCAGTAGTT			
Tert	F: GACCTCCATCAGAGCCAGTC	60	84	RT-PCR
	R: CGCAAGACCCCAAAGAGTT			
Tert	F: GCGTTTGGTGGATGATTTCT	60	254	QPCR
	R: AGCTGGAGTAGTCGCTCTGC			
Differentiation assay genes				
AFP	F: TGCGTTTCTCGTTGCTTACA	60	81	RT-PCR
	R: GCTGCCATTTTTCTGGTGAT			
AFP	F: Acacaaaaagcccactccag	60	147	QPCR
	R: ggtgcatacaggaagggatg			
DBH	F: tgactgggagaaaggtggtc	60	160	QPCR
	R: tacgtgcaggaggtgatgag			
CAC	F: tgctgatcgtatgcagaagg	60	135	RT-PCR QPCR
	R: gctggaaggtggacagagag			

8. Absolute ethanol (Merck, Darmstadt, Germany).

9. Standard table-top microcentrifuge capable of $13,000 \times g$ centrifugal force.

10. Standard thermal cycler.

11. NanoDrop spectrophotometer or similar to evaluate the synthetized cDNA quality.

2.7.4 Telomerase Activity

1. TRAPEZE Telomerase Detection Kit (Chemicon, Billerica, MA).

2. DPBS without Ca^{2+}, Mg^{2+} (GIBCO).

3. 10–20 % nondenaturing polyacrylamide precasted gels.

4. 10× TBE (BioRad, Hercules, CA).

5. SYBR green (Molecular Probes).

6. Loading buffer $10\times$ (BioRad).

7. Nondenaturing 10–20 % polyacrylamide gel (15 % precasted gels from BioRad).

3 Methods

Extracellular matrix compounds play important roles in cell adhesion, attachment, cell interactions, and proliferation (18, 11). Previous studies have demonstrated that the components of ECM support undifferentiated growth of hESCs. Extracellular matrix is generally organized into a three-dimensional fibrous structure and therefore, it is crucial to extract the ECM samples from hFFs rapidly and at 4 °C which prevents conformational changes and protein degradation. Transmission electron microscopy (TEM) and atomic force microscope (AFM) can be used to assess the integrity of hffECM after the obtention. To validate functionality, it is very important to evaluate the ability of the extracted ECM to maintain hESCs in the undifferentiated state. This can be assessed by long-term growth of hESCs on the hffECM and by routine analysis of pluripotency using the following assays: RT-PCR, QPCR, immunocytochemistry, determination of alkaline phosphatase, and telomerase expression and analysis of spontaneous differentiation ability (19).

3.1 Preparation of Fibroblast Cells and Conditioned Medium

1. hFFs are grown in Iscove's Medium and the cells are split using Tryple Select every 5–7 days.

2. When confluent, the cells were inactivated using mitomycin C at 37 °C in an incubator with 5 % CO_2 for 3 h.

3. They are then washed with DPBS three times (5 min), digested, and counted (*see* **Note 2**).

4. Seed fibroblast at a density of 6×10^6 cells in a T75 flask coated with 0.1 % gelatine, and culture at 37 °C with 5 % CO_2 for 24 h before adding TERS1 medium.

5. TERS1 conditioned medium is collected every day until day 7, and stored at −80 °C for up to 6 months (*see* **Notes 3** and **4**).

3.2 Preparation of Extracellular Matrix (ECM)

1. hFFs are grown in an appropriate medium and the cells are split using Tryple Select every 5–7 days. When the cells reach 100 % confluence, they are inactivated by treatment with mitomycin C (10 µg/mL) for 3 h, and then washed three times with DPBS.

2. Cells are detached, counted, and seeded (2×10^5 cells/per well) in a six-well plates, coated with 0.1 % gelatin, and cultured at 37 °C and 5 % CO_2 for 24 h.

3. Inactivated cell cultures are maintained for 7–8 days, with a medium change every second day.

4. Foreskin cultures are treated on day 7, following the recommendations described in (15).

5. Cells are washed twice with DPBS without Ca^{2+} and Mg^{2+} and subsequently lysed by osmotic shock with Tris–EDTA buffer (10 mM Tris, 1 mM EDTA, pH 7.4) adding 1.5 mL per 10^6 cells.

6. After addition of osmotic shock buffer, cells are incubated o.n. at 4 °C using an orbital shaker, after which time the Tris–EDTA buffer is removed by aspiration (*see* **Note 9**).

7. Lysed cells are eliminated from the plates by rinsing six times (5 min each) with DPBS. Plates containing the remaining hffECM are stored at 4 °C or dried and sterilized using standard methods. Examples of hFF cells and ECM samples studied using TEM are shown in Fig. 1a.

3.3 Preparation of Samples for TEM (See Notes 6 and 10 When Planning These Experiments)

1. Wash samples twice with DPBS for 2–3 min.

2. Fix for 30 min at 37 °C with 3 % glutaraldehyde. Add glutaraldehyde carefully until the plate surface is totally covered.

3. Incubate for 30 min and store at 37 °C, keeping the samples inside a box or Petri dish to avoid release of carcinogenic vapor.

4. Aspirate glutaraldehyde with a disposable Pasteur pipette and discard adequately.

5. Wash 3–4 times for 3 min with PBS.

6. Cover the samples with DPBS and maintain them at 4 °C (*see* **Note 11**).

7. Fixation and contrasting: add 1 mL of 2 % osmium to cover the plates and leave for 1 h.

8. Wash three times (5 min each) with distilled water at 4 °C (do not exceed 15 min).

9. Dehydration procedure (all steps are performed at 4 °C):

   (a) Add 30 % alcohol for 5 min.

   (b) Wash with 50 % alcohol (10 min) at 4 °C (exceeding this time can cause protein damage).

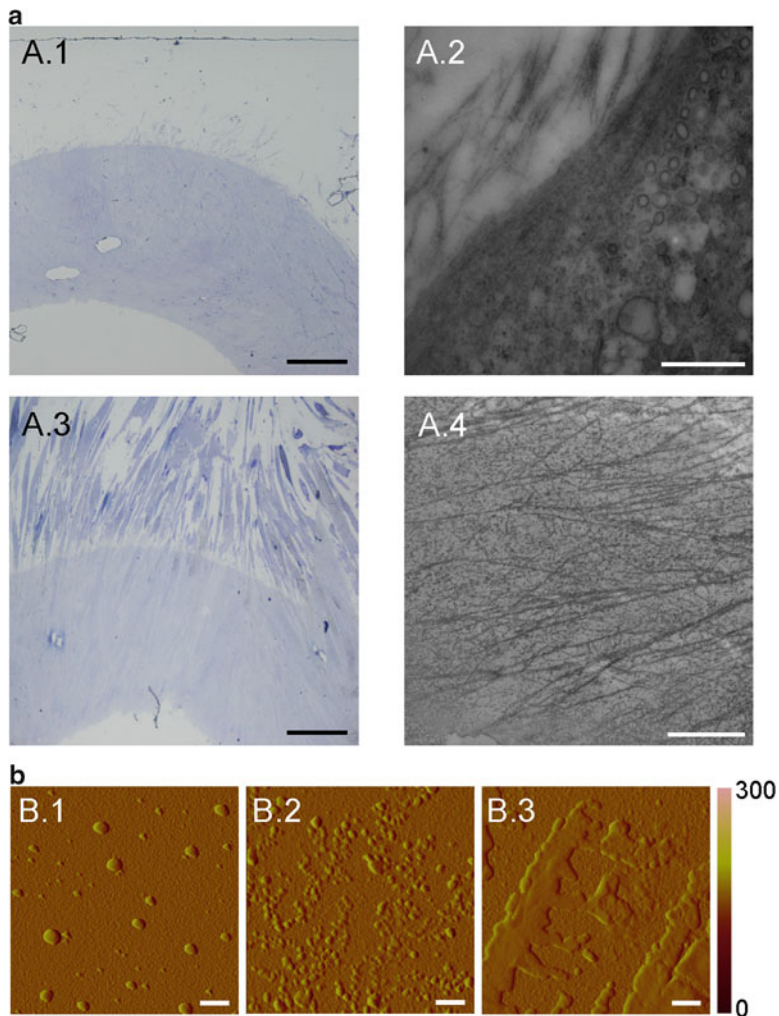   (c) Wash twice with 70 % alcohol (10 min each).

Fig. 1 Transmission electron microscopy (TEM) and atomic force microscopy (AFM) of the hffECM after extraction to evaluate structural integrity and surface roughness. (**a**) Ultrastructural analysis of the efficiency of the extraction protocol using TEM. Plates of lysed cultures and their respective controls with intact hFFs were fixed and processed for TEM ultrastructural analysis. hFFs were maintained for the indicated times and then subjected to hypotonic lysis. (**a1** and **a3**) Semi-thin sections were prepared from control and lysed cultures and were stained with toluidine blue. (**a2**) Representative TEM image of intact hFF control cells after 7 days in culture. (**a4**) Representative TEM image of lysed culture plate after 7 days in culture. Scale bars, 200 μm (**a1** and **a3**), 1 μm (**a2**), 500 nm (**a4**). (**b**) AFM analysis of ECM conformation on glass surfaces. The images show the amplitude mode on the same scale. (**b1**) The lowest surface roughness value corresponds to fibronectin that was used as experimental control. The mean surface roughness observed demonstrates that the surface roughness is significantly lower on day 1 (**b2**) than after hFFs culture on day 2 (**b3**). Scale bars 250 nm. (Figures **a1** and **a3** reproduced from (12) with permission from Springer)

10. Add 1 mL of 2 % uranyl acetate in 70 % alcohol and incubate for 2 h at 4 °C.

11. Continue with the dehydration process by using the following alcohol gradient:

    (a) Add 70 % ethanol 2 times × (10 min each).

    (b) Wash with 90 % ethanol 2 times × (10 min each).

12. To embed the samples in resin, use the following alcohol/LR white resin (EMS) gradient as follows:

    (a) Incubate in 2 parts 90 % ethanol: 1 part resin for 45 min at RT.

    (b) Replace the mixture using a Pasteur pipette and discard adequately.

    (c) Prepare a dilution 1 part 90 % ethanol: 2 parts resin, add to the samples and incubate for 45 min.

    (d) Remove the mixture and discard appropriately.

    (e) Mix 1 part 100 % ethanol and 2 parts resin and incubate for 45 min.

    (f) Replace the mixture by adding 100 % LR white resin. Incubate o.n. in an orbital shaker.

13. To allow polymerization, transfer the samples to an oven and incubate them at 60 °C for 24 h.

14. Detach and proceed with the sectioning.

15. Observe the samples under a transmission electron microscope.

3.4   Atomic Force Microscopy (AFM)

AFM provides detailed 3-dimensional images of ECM protein–surface interactions.

1. Samples should be washed in 0.1 M DPBS and fixed in 2 % paraformaldehyde/2.5 % glutaraldehyde/PB for 1 h at room temperature.

2. Samples are dehydrated using a series of ethanol/water solutions (30 %, 50 %, 70 %, 90 %, and 96 %) for 10 min with final dehydration in absolute ethanol for 10 min twice.

3. The dehydrated samples were examined by AFM, operating in the tapped-air mode.

4. The ECM protein distribution (on glass) is estimated from AFM images. The amplitude set point should be 1.3 V with a drive amplitude of 300 mV.

5. Surface roughness is calculated by processing 10 representative AFM images.

Images of glass surfaces with and without hffECM captured using AFM are shown in Fig. 1b.

3.5 Growth of Undifferentiated Pluripotent Cells Using Feeder-Free Conditions and hffECM

1. Once the ECM-coated plates are ready for use, conditioned medium which has been collected, should be added.

2. Add 2 mL of conditioned medium containing 50 ng/mL and 100 ng/mL of fresh TGF-β1 and bFGF, respectively. Place the 6-well plates in an incubator at 37 °C with 5 % CO_2 for at least 3 h before seeding the new hESC colonies.

3. Wash the hESC colonies maintained on human feeders twice with prewarmed DMEM medium.

4. Dissect hESC colonies mechanically and collect them in a 15 mL polypropylene tube (*see* **Notes 12** and **13**).

5. Centrifuge the cells for 3 min at $200 \times g$ and remove the supernatant.

6. Gently resuspend the pellet in conditioned medium supplemented with fresh TGF-ß1 and bFGF in the same 15 mL tube.

7. Add 1 mL of medium containing 10–20 colonies to each well of the ECM-coated plates.

8. Incubate the plates at 37 °C with 5 % CO_2 and change the medium every second day.

9. Undifferentiated hESCs should be transferred to new plates every 5–7 days and maintained at 37 °C with 5 % CO_2.

Examples of contrast phase microscope are shown in Fig. 2a, b.

3.6 Detection of Cell Surface Pluripotency Markers by Immunocytochemistry

1. Remove the culture medium.

2. Wash the cells once with DPBS without Ca^{2+} and Mg^{2+}. It is important to add the DPBS very gently and not directly to the cells.

3. Fix hESCs in 4 % paraformaldehyde for 15 min at room temperature by adding 1.5 mL/well to the 6-well plates.

4. Wash 4 times with DPBS.

5. To permeabilize the hESCs, add 1.5 mL per well of 1 % Triton-X-100, followed by an incubation of 10 min at room temperature.

6. Wash twice (5 min each) with DPBS.

7. Apply 4 % of blocking solution at room temperature for 45 min. Serum for the blocking solution should be of the same species as the secondary antibody.

8. Remove the blocking solution but do not wash the cells.

9. Dilute the primary antibody blocking solution (*see* Table 1).

10. Add 1 mL of the corresponding primary antibody dilution to the cultures for at least 1 h at RT or overnight at 4 °C.

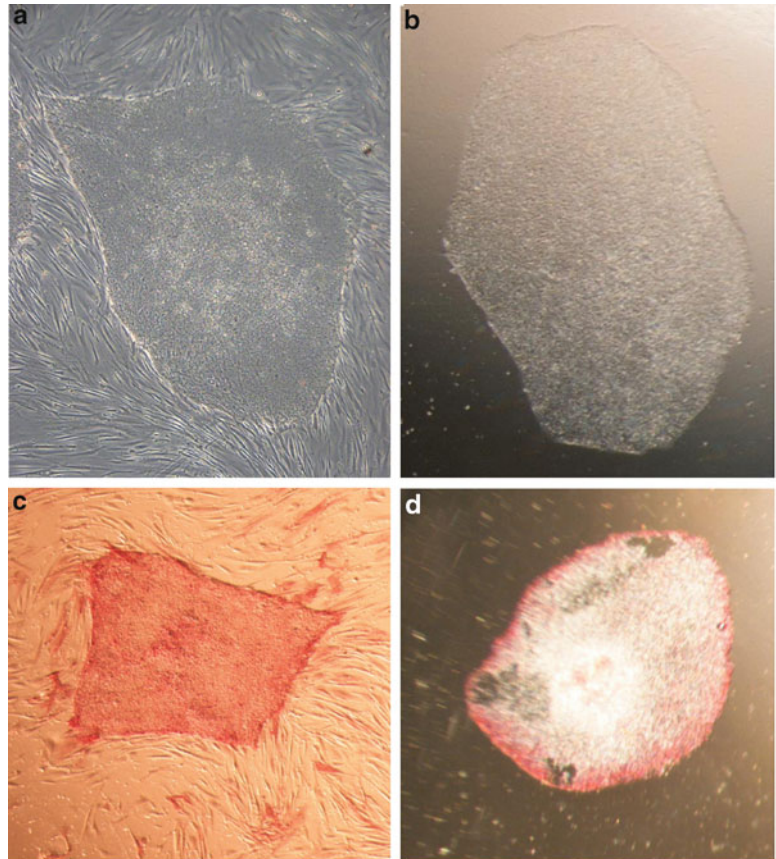11. Wash 3 times with DPBS. Cells can be left overnight before adding the secondary antibody.

Fig. 2 (**a** and **b**) Morphology of undifferentiated hESCs grown for 11 passages over feeder cells and on plastic coated with hffECM using conditioned medium respectively. (**c** and **d**) Images showing alkaline phosphatase activity in both feeder and hffECM conditions, indicating maintenance of pluripotency. Images were obtained using Zeiss Axiovert 200 M microscope (magnifications ×100)

12. Dilute the secondary antibody in DPBS and add 1 mL per well (*see* Table 2).

13. Incubate at room temperature for 60 min and in darkness as exposure to light may cause bleaching of fluorescent labels.

14. Wash the cells 4 times with DPBS.

15. If the cells are attached to a coverslip mount it on a slide using prolong gold antifade reagent with DAPI. Remove any bubbles that may have formed during mounting.

16. Let the slides dry for 15 min in conditions where they are protected from light.

17. After 90 min the samples can be observed using a fluorescence microscope. Examples of the morphology and undifferentiated hESC surface markers grown on plastic dishes coated with ECM derived from hFFs and in the presence of conditioned medium are presented in Figs. 2c, d, and 3.
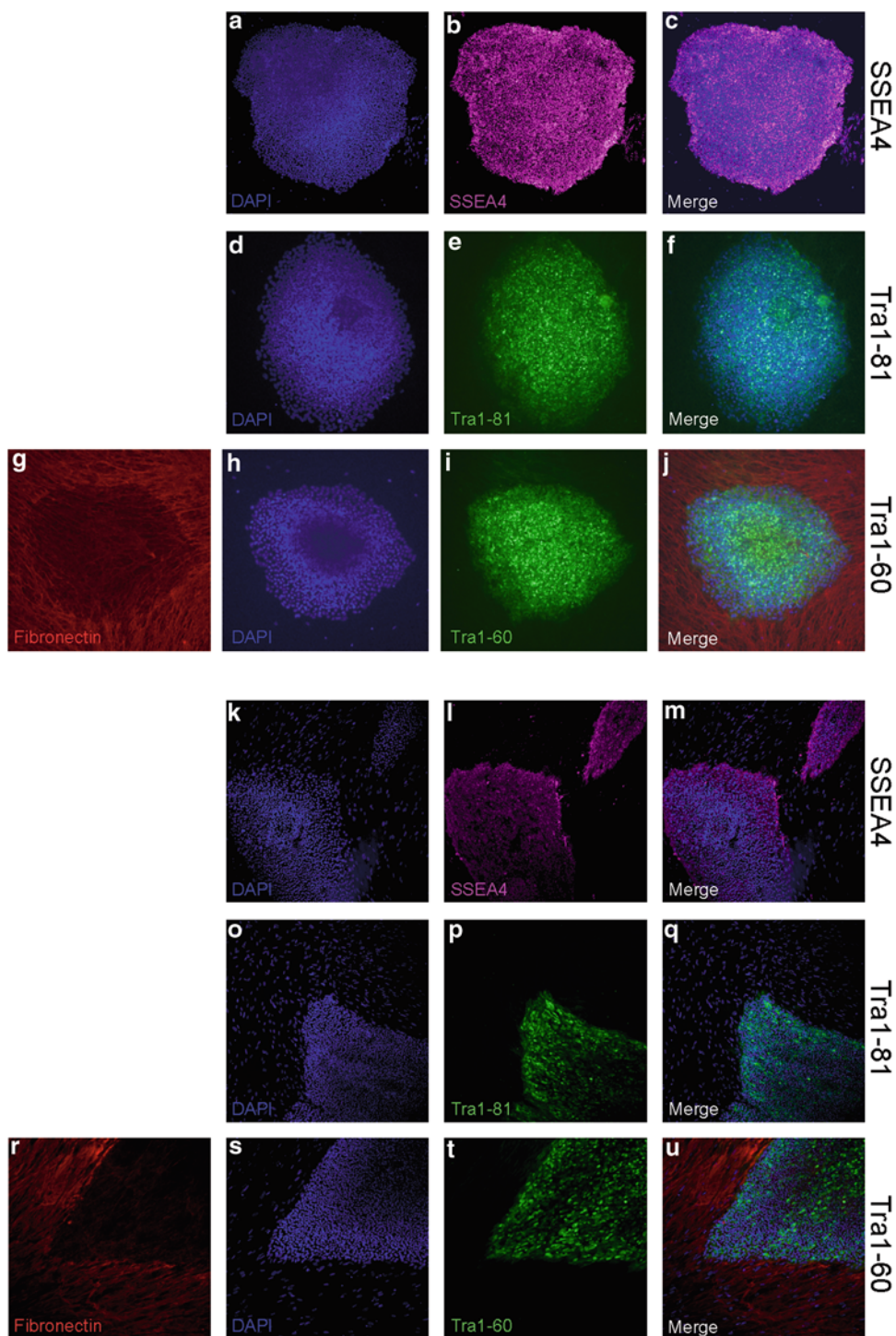
Fig. 3 Morphology and cell surface markers of undifferentiated hESCs grown for 11 passages on plastic dishes coated with hffECM derived from hFFs and in the presence of conditioned medium. Note round and compacted hESC colonies. The presence of SSEA4 (**a**–**c**, *magenta*), Tra1-81 (**d**–**f**, *green*) Tra1-60 (**h**–**j**, *green*), pluripotency markers were observed. Note the presence of specific fibronectin staining (**g** and **j**). Morphology and cell surface markers of undifferentiated hESCs grown for 11 passages over hFFs feeder. The presence of SSEA4 (**k**–**m**, *magenta*), TRA-1-81 (**o**–**q**, *green*), TRA-1-60 (**s**–**u**, *green*) pluripotency markers were observed. Note the presence of specific fibronectin staining (**r** and **u**). Images were obtained using confocal fluorescence microscope (×10 magnifications)

3.7 Preparation of Samples for RT-PCR and QPCR Analysis

Analyzes of mRNA expression by reverse transcription PCR (RT-PCR) and real-time quantitative PCR (QPCR) are carried out using standard protocols. An RT-PCR reaction is performed at 60 °C and for 35 cycles, except for ß2-microglobulin (performed at 55 °C for 35 cycles). The QPCR reaction is performed at 60 °C (60 s) and 95 °C (15 s) for 40 cycles. Primer sequences were designed using Primer3 software and synthesized by Sigma-Aldrich. All primer sequences and DNA fragments are listed in Table 3. Transcripts encoding the following proteins Oct-4, Tert, Nanog, and FoxD3 should be assessed for pluripotency, and AFP, DBH, and CAC for differentiation (specific lineage) markers. The ß2-microglobulin gene is used as an endogenous control (housekeeping) gene.

3.7.1 Extraction and Quantification of Total RNA

Total RNA extraction can be performed using 20 hESC colonies maintained on ECM. As a positive control, undifferentiated hESCs maintained on feeder cells should be used. We use the RNeasy mini kit (74104; Qiagen) following provider-recommended instructions with some modifications. To eliminate contamination by genomic DNA, the initial RNA pellet was incubated with deoxyribonuclease (DNase) I (RNase-free DNase SET; 79254; Qiagen).

1. Cut the cells from the culture dish with a needle and collect them in a centrifuge tube.

2. Add 350 μL of buffer RLT and vortex for 1 min.

3. Centrifuge the lysate for 3 min at maximum speed. Carefully remove the supernatant by pipetting out and discarding it.

4. Add 1 volume of 70 % ethanol to the homogenized sample and mix well by pipetting only (do not centrifuge). The volume might be less than 350 μL due to loss during the homogenization process. The use of ethanol can trigger precipitates to form; however, their presence has no impact on the process.

5. Transfer up to 700 μL from the sample, including any precipitates, to an RNeasay mini spin column (pink) placed in a 2 mL collection tube, and close the lid.

6. Centrifuge at 8,000 × g (10,000 rpm) for 15 s, discard the flow-through and reuse the collection tube for step 7. Note: if the sample volume is greater than 700 μL, the aliquots must be centrifuged one after the other in the same column, discarding the flow-through after each centrifugation.

7. Add 350 μL RW1 buffer to the RNeasy mini spin column, close the lid, and centrifuge at 8,000 × g (10,000 rpm) for 15 s. Discard the flow-through and reuse the collection tube for step 10.

8. Add 10 μL DNase I stock solution to 70 μL RDD buffer. Mix by gently inverting the tube; centrifuge briefly to collect the liquid from the tube walls.

9. Add DNase I to the incubation mix (80 µL) and transfer it directly to the RNeasy column membrane, and it place on the benchtop at 20–30 °C for 15 min.

10. Add 350 µL RW1 buffer to the RNeasy mini spin column, close the lid, and centrifuge at $\geq 8,000 \times g$ for 15 s. Discard the flow-through.

11. Add 500 µL RPE buffer to the RNeasy mini spin column, close the lid, and centrifuge at $\geq 8,000 \times g$ for 15 s. Discard the flow-through and reuse the collection tube.

12. Add 500 µL RPE buffer to the RNeasy mini spin column, close the lid, and centrifuge at $\geq 8,000 \times g$ for 2 min to wash the column membrane.

13. Place the RNeasy mini spin column in a new 2 mL collection tube and centrifuge at full speed for 1 min to dry the membrane (optional step).

14. Place the RNeasy mini spin column in a new 1.5 mL collection tube and add 30–50 µL RNase-free water directly to the spin column membrane. Close the lid, and centrifuge at $\geq 8,000 \times g$ for 1 min to elute the RNA.

15. If the expected RNA yield is >30 µg, repeat step 14 using another 30–50 µL RNase-free water, or the elute from step 14 (if a high RNA concentration is required). Reuse the collection tube from step 14. Note: if the elute from step 14 is used, the RNA yield might be 15 % lower than using a second volume of RNase-free water but the final RNA concentration will be higher (*see* **Note 14**).

16. Quantify the extracted RNA and evaluate its quality using a NanoDrop spectrophotometer or a RNA-integrity gel. Either use the eluted RNA directly in following steps (RT-PCR and QPCR) or store the eluted RNA at −80 °C for later analysis.

3.7.2 cDNA Synthesis   Use 50 µL from each sample obtained for total RNA to single-stranded cDNA reverse transcription (RT). We follow the recommendations from the High Capacity cDNA RT kit (PN 4368814, Applied Biosystems).

1. Place the 2× RT master mix on ice and mix gently.

2. Pipette 25 µL of 2× RT master mix into each well of an individual tube (*see* **Note 15**).

3. Pipette 25 µL of RNA sample into each well, pipetting up and down two times to mix (*see* **Note 16**).

4. Seal the tubes and briefly centrifuge them to spin down the contents and to eliminate any air bubbles.

5. Place the plate or tubes on ice until you are ready to load them into the thermal cycler.

6. Program the thermal cycler conditions with the following parameter steps: (1) step 1: 25 °C for 10 min; (2) step 2: 37 °C for 120 min; (3) step 3: 85 °C for 5 min; (4) step 4: 4 °C for indefinite time.

7. Set the reaction volume to 20 μL.

8. Load the reactions into the thermal cycler and start the reverse transcription run.

9. Quantify the synthetized cDNA and evaluate its quality using a NanoDrop spectrophotometer or cDNA integrity gel.

3.7.3 PCR Analysis

Use a 50 μL reaction per sample to obtain the PCR products. We follow the recommendations from FastStart PCR Master (04710436001; Roche).

1. Prepare the microplates for PCR according to the instrument instructions.

2. Thaw the solutions and briefly spin the vials in a microcentrifuge before opening.

3. Mix the solutions by pipetting them up and down gently and store them on ice.

4. Prepare 10× concentration solutions of the PCR primers (*see* Table 3)

5. Prepare a PCR mix for the number of reactions to be run plus one additional reaction. The PCR mix contains the following products for each reaction in the following order: 25 μL 2× master mix, 5 μL forward primer (3 μM), 5 μL reverse primer (3 μM), and 10 μL PCR-grade water.

6. Mix the solution by pipetting it up and down (do not vortex).

7. Transfer 45 μL PCR mix into each reaction well of the PCR microplate.

8. Add 5 μL of template DNA (up to 100 ng cDNA) into each reaction well.

9. Prepare the microplate for the PCR reaction according to the instrument's instructions.

10. Program the thermal cycler conditions with following steps: (1) step 1: 1 cycle at 95 °C for 4 min; (2) step 2: 30–40 cycles at 95 °C for 30 s, and 45–65 °C for 30 s, and 72 °C for 45–180 s; (3) step 3: 1 cycle at 72 °C for 7 min; (4) step 4: 4 °C for an indefinite time.

11. Set the reaction volume to 50 μL.

12. Load the microplate into the thermal cycler and start the PCR reaction.

The results can be observed in the electrophoresis gels on Fig. 4b, c.
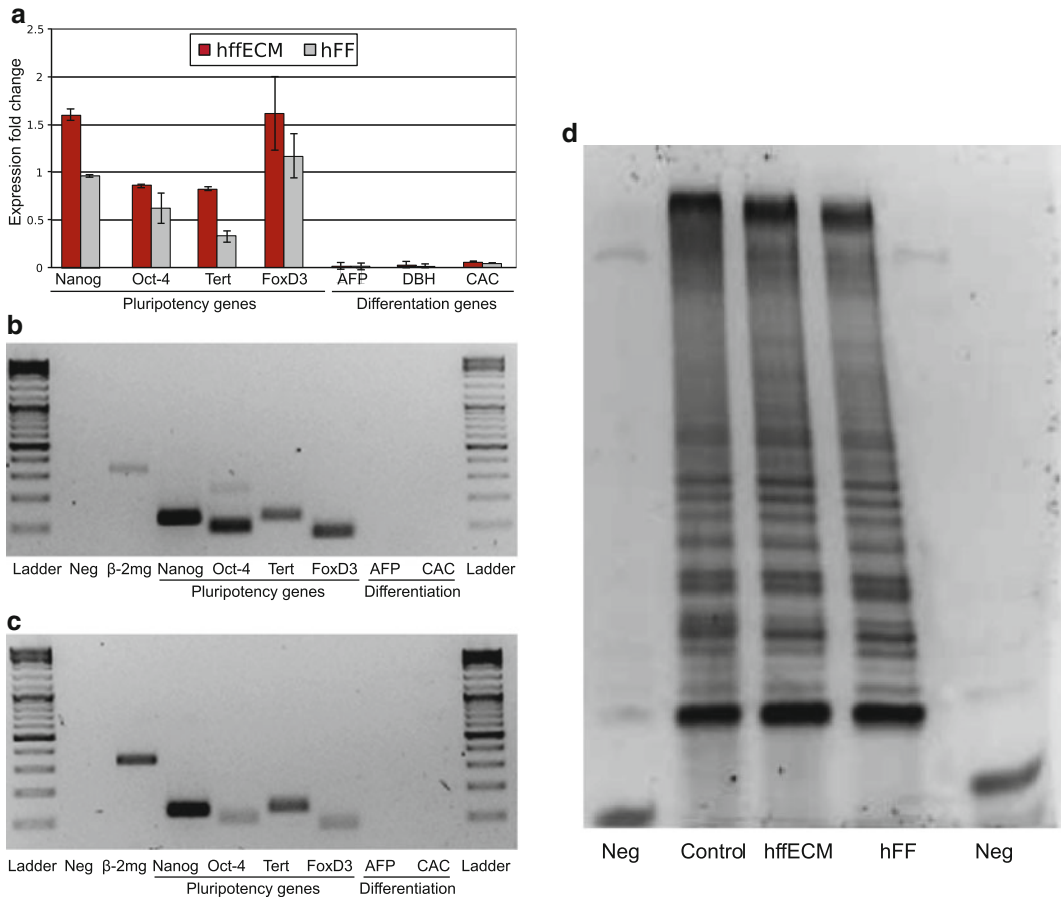
Fig. 4 Analysis of specific molecular hESC markers and telomerase activity. hESCs were grown on hffECM and in the presence of conditioned medium for 11 passages. Controls over foreskin feeder were also grown at the same time. (**a**) QPCR comparing expression of pluripotency and differentiation genes in cells maintained on hffECM and hFF. The expression of characteristic pluripotent markers is higher in the cells maintained on hffECM than on hFF. This indicates that our system is highly selective and only the cells with the best quality are able to adapt and survive. No expression of differentiation markers (AFP, DBH, and CAC) was detected. (**b** and **c**) Cells maintained on hffECM and foreskin feeders respectively. Line 1 and 10: Ladders with 1 Kb; 2. Corresponds to negative control; 3. Housekeeping gene ß-2 microglobulin. Cells express all the typical hESC markers 4. Nanog; 5. Oct-4; 6. Tert; 7. FoxD3. After 11 passages on ECM cells were negative for differentiation markers AFP and CAC (lines 8 and 9, respectively). (**d**) Colonies retained telomerase activity after 11 passages on hffECM (line 3). Line 4 corresponds to their H9 counterparts maintained 11 passages over foreskin feeder cells. Line 1 and 5 are negative controls, and line 2 the positive control

3.7.4 QPCR Analysis

1. Completely thaw the Power SYBR Green PCR Master Mix and gently mix the reagents.

2. In polypropylene tubes, prepare the PCR reagent mix for the desired number of PCR reactions. Each 20 μL PCR reaction contains 10 μL of the PCR Master Mix, 50–300 nM from each primer, 100 ng template, and a variable quantity of nuclease-free water (*see* **Note 17**).

3. Mix gently (do not vortex) and centrifuge briefly.

4. Prepare the microplate for the QPCR reactions according to instrument's instructions.

5. Program the thermal cycler conditions with the following steps: (1) step 1: 1 cycle at 95 °C for 4 min; (2) step 2: 30–40 cycles at 95 °C for 30 s, 45–65 °C for 30 s, and 72 °C for 45–180 s; (3) step 3: 1 cycle at 72 °C for 7 min; (4) step 4 at 4 °C for an indefinite time.

6. Set the rest of the thermal cycler parameters (e.g., volume = 20 μL) following instrument user's manual to configure the plate documentation details.

7. Load the microplate into the thermal cycler and start the PCR reaction.

8. Analyze and export the results using a spread sheet program with statistical features.

9. Calculate the average cycle time, the standard deviation, and the coefficient of variation for each group of replicates of any sample. Remove outlier points (Ct > 0.3 × standard deviation).

10. Apply the ΔΔCt method to obtain "fold changes" as desired output ([20]). The internal control selected is ß-2 microglobulin gene.

3.8 Telomerase Activity Assay

Telomerase activity is assayed using telomeric repeat amplification protocol using the Trapeze Kit (Chemicon) and according to the manufacturer's protocol but with some modifications.

3.8.1 Sample Preparation

1. For stem cell analysis, collect 30–100 colonies. Pellet cells at 400 × g for 5 min at 4 °C. We highly recommend that the positive and negative controls provided with the kit and a negative control (a differentiated cell line and/or heat inactivated immortal/stem cells) be used.

2. Wash cell pellet with sterile Ca^{2+} and Mg^{2+} free DPBS for 5 min at 400 × g at 4 °C.

3. Resuspended the cell pellet with 5–20 μL CHAPS lysis buffer (Thermo Scientific) for stem cell colonies.

4. Place on ice for 30 min.

5. Pellet cells at 9,500 × g (12,000 rpm) at 4 °C for 20 min.

6. Transfer the supernatant to a new tube. At this point the supernatants are kept at −80 °C and can be stored for up to 1 year to be used for telomerase detection.

3.8.2 PCR Reaction

1. Use 2–4 μL of sample per PCR reaction.

2. Heat inactivated negative controls must be subjected to 85 °C for 10 min. Then use the same 2–4 μL volume for the PCR reactions.

3. Prepare a master mix containing all these components except the templates, all the reagents are provided by the kit except Taq polymerase:

10× TRAP reaction buffer	5 µL
50× dNTP mix	1 µL
TS primer	1 µL
TRAP primer mix	1 µL
Taq polymerase (5 U/µL)	0.4 µL
dH$_2$O	29.5 µL
Template	2 µL

4. PCR program.

1 cycle:	30 °C 30 min
30–33 cycles:	94 °C 30 s
	59 °C 30 s

3.8.3 Separation by Electrophoresis

1. For sample electrophoresis, use a 10–15 µL PCR reaction with a 2–5 µL loading buffer 10× (BioRad).

2. Load a nondenaturing 10–20 % polyacrylamide gel (we use 15 % precasted gels from BioRad).

3. Run the gel in TBE 0.5× until both color bands are out of the gel.

4. Prepare the SYBR green solution (1/10,000) in TBE 1×.

5. Stain the gel for 15–20 min in the dark.

6. Visualize the bands in a transilluminator (with the same wavelength as the ethidium bromide). The results for telomerase activity are presented in Fig. 4d.

4   Notes

1. Conditioned medium can be prepared using hFFs between passages 11–18, but in order to maintain the quality, we recommend that cells between passages 11–16 be used.

2. Cell counting: take 20 µL of cell suspension and dilute it with 70 µL of culture medium. Add 10 µL of Trypan Blue solution (Sigma), mix, and incubate for 1 min before counting viable (round, clear cells) and nonviable (blue) cells using a Neubauer haemocytometer and plate 6×10^6 cells per flask or 2×10^5

cells per well for 75 cm^2 flasks and 6-well plates, respectively. More than 75 % of cells should be viable.

3. Before using of conditioned TESR1 medium, add 100 ng/mL of bFGF and 50 ng/mL of TGF-β1. Conditioned medium can be kept at +4 °C for 1 week or stored at −80 °C for 6 months.

4. No differences between frozen and fresh conditioned medium have been observed.

5. Lysis buffer preparation. The following is an example for 250 mL:

    (a) 2.5 mL 1 M Tris–HCl pH 7.4

    (b) 500 μL 0.5 M EDTA pH 8.0

    (c) 247 mL H$_2$O

    Stock Solutions:

    • 1 M Tris–Cl, pH 7.4 (1 L):
        – 121 g Tris Base
        – 1 L H$_2$O
          Adjust pH

    • 0.5 M EDTA (1 L):
        – 186.1 g EDTA Na$_2$H$_2$O
        – 1 L H$_2$O
          Adjust pH

6. TEM inclusion procedure: Glutaraldehyde is carcinogenic by inhalation, it is important to manipulate it in a fume hood. During the 37 °C incubation it is very important to avoid inhaling these fumes, keeping the culture plates inside a petri dish in the special stove. Osmium is carcinogenic and toxic by inhalation and contact and so double gloves and a mask should be worn, in addition to working under fume hood. Uranyl acetate is carcinogenic and radioactive. Manipulation must be performed in an adequate installation under fume hood using double gloves and a mask. Given their toxicity, those residues need to be manipulated and discarded properly.

7. Try to avoid bubbles in the samples.

8. Do not use until the solution reaches room temperature and adjust the pH if necessary.

9. We have also performed this process by incubating with ice in the orbital shaker for 2.5 h.

10. When planning to do TEM analysis, the seeding of hFFs, the cell lysis, and subsequent pluripotent cell seeding needs to be done on Permanox® chambers.

11. At this point, the plates can be maintained for several days at 4 °C by adding sodium azide.

12. hESCs were cultured and maintained as previously described (21). Cells were passaged mechanically and replated on ECM-coated plates.

13. The optimal size of hESCs before the splitting procedure is when the colonies completely cover the magnification field (10×).

14. If you use the elution obtained in step 10, the quantity of RNA obtained may be 15–30 % less than using RNase-free water.

15. The kit reagents must always be thawed on ice.

16. Use up to 5 μg of total RNA per 50 μL RT reaction.

17. Include an extra volume to account for pipetting losses.

Acknowledgements

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Efficient Expansion of Dissociated Human Pluripotent Stem Cells Using a Synthetic Substrate

Eihachiro Kawase

Abstract

Human pluripotent stem cells (hPSCs), including human embryonic stem cells and human-induced pluripotent stem cells, are a renewable cell source for a wide range of applications in regenerative medicine and useful tools for human disease modeling and drug discovery. For these purposes, large numbers of high-quality cells are essential. Recently, we showed that a biological substrate, recombinant E8 fragments of laminin isoforms, sustains long-term self-renewal of hPSCs in defined, xeno-free medium with dissociated single-cell passaging. Here, we describe a modified culture system with similar performance to efficiently expand hPSCs under defined, xeno-free conditions using a non-biological synthetic substrate.

Keywords: Human embryonic stem cells, Synthetic substrate, Defined culture, Xeno-free culture, Culture substrate

1   Introduction

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human-induced pluripotent stem cells, have a self-renewal ability and pluripotency to differentiate into all three germ layers in vitro and in vivo (1–3). Because of these notable properties, hPSCs are expected to be valuable tools for studies of human development, diseases, and drug discovery as well as a cell source for regenerative medicine. In general, clinical applications of stem cells may require up to $1 \times 10^{9-10}$ cells per patient (4, 5). Furthermore, functional disease modeling efforts typically require $>1 \times 10^6$ cells to generate a single differentiated cell type (6). Therefore, robust methods for scalable expansion of hPSCs are critical for these purposes.

Traditional hPSC culture methods require the use of mouse or human fibroblast feeder layers or feeder-conditioned medium. These culture methods are labor intensive and hard to scale up. In addition, it is difficult to maintain undifferentiated hPSCs because of the undefined conditions. hPSCs usually propagate as colonies in culture, and their passaging involves either enzymatic dissociation with gentle pipetting or manual microdissection of hPSC colonies

at an appropriate size to sustain an undifferentiated state. Formation of large aggregates during passaging accelerates their spontaneous differentiation, whereas complete dissociation of hPSC clumps into single cells causes extensive cell death (<1 % survival) (7, 8). As a result, conventional colony-based protocols often have considerable variations in culture outcomes because of the difficulty to precisely control dissociation of hESCs while passaging. We believe that a method to overcome such difficulties might be identification of an appropriate culture substrate for hPSCs to enable complete dissociation of hPSCs by an optimized and standardized protocol.

We have recently reported long-term undifferentiated culture of dissociated hPSCs using recombinant E8 fragments of laminin isoforms (LM-E8s) (9). hPSC lines are well maintained on LM-E8s in chemically defined, xeno-free medium for more than 30 passages with single-cell passaging. The hPSCs sustain high expression of pluripotency markers, a normal karyotype, and the potential for differentiation into all three germ layers. However, biological materials are generally expensive to manufacture, have limited scalability, and may have batch-to-batch variability. Therefore, in this study, we examined synthetic substrates to replace LM-E8s.

Here, we show that a synthetic peptide-acrylate surface, Synthemax, sustains long-term self-renewal of hPSCs in defined, zeno-free medium with dissociated single-cell passaging. Synthemax is an acrylate polymer modified with an amino-containing peptide, which has been shown to be effective for prolonged culture of multiple hESC lines (10). However, Synthemax has been only used for propagation of hPSC as colonies in culture and not completely dissociated cells. Synthemax does not provide stronger cell adhesion or migratory abilities than those of LM-E8s. Therefore, we added the ROCK inhibitor Y-27632 for 1 day of incubation during passaging. This modification shows similar performance as hPSC culture on LM-E8s.

2 Materials

2.1 Human Pluripotent Cell Lines

hESC lines: KhES-1, KhES-3, and H9 (WA09).

2.2 Culture Medium

1. Defined grade (*see* **Note 1**)
   mTeSR1 (STEMCELL Technologies, cat. no. ST-05850).
   We did not test other defined commercial media.

2. Xeno-free grade (*see* **Note 2**)
   TeSR2 (STEMCELL Technologies, cat. no. ST-05860).
   NutriStem hESC XF (Stemgent, cat. no. 05-100-1).
   PSGro (StemRD, cat. no. SC500M-1).

| *2.3*   *Culture Vessel* | Synthemax Surface 6-well plates (Corning, cat no. 3979) (*see* **Note 3**). |

2.4   *Cell Dissociation Reagents*

1. Phosphate-buffered saline (PBS) (Sigma, cat. no. D-5652).
2. 0.2 and 0.02 % EDTA/PBS (Sigma, cat. no. E8008) (*see* **Note 4**).
3. TrypLE Select (Invitrogen, cat. no. 12563-011) and accutase (Innovative Cell Technologies, cat. no. AT104) (*see* **Note 5**).

2.5   *ROCK Inhibitor*

10 mM Y-27632 (Wako, 275-00513; Tocris, #1254; Sigma, Y-0503) (*see* **Note 6**).

2.6   *Cryopreservation Medium (See Note 7)*

| Complete medium | 0.8 mL |
| Dimethyl sulfoxide | 0.2 mL |

2.7   *Additional Materials*

For flow cytometric analysis, we assessed the undifferentiated state of cells using the following kits: Human Pluripotent Stem Cell Sorting and Analysis Kit (BD Stemflow, #560461), Human Pluripotent Stem Cell Transcription Factor Analysis Kit (BD Stemflow, #560589), and Human And Mouse Pluripotent Stem Cell Analysis Kit (BD Stemflow, #560477). An anti-TRA-1-60 antibody is not included in these kits. Therefore, we purchased an anti-TRA-1-60 antibody (PE-conjugated, BD Bioscience, #560193) (*see* **Note 8**).

3   Methods

3.1   *Passaging of hPSCs in Xeno-Free Medium (See Note 9)*

1. Prior to starting, equilibrate solutions to room temperature. Prepare Synthemax II-SC substrate by coating 6-well plates according to the manufacturer's instructions.
2. Aspirate culture medium from the vessel.
3. Rinse with 3 mL PBS twice.
4. Add 1 mL of 0.2 % EDTA/PBS and incubate for 2–5 min at room temperature (Fig. 1a) (*see* **Note 4**).
5. Aspirate the EDTA/PBS.
6. Add 1 mL TrypLE Select, aspirate it, and incubate for 1 min at 37 °C (Fig. 1b). Accutase may be used as a substitute. Incubate for 3–10 min at room temperature or 3–5 min at 37 °C (Fig. 1c). For PSGro medium, TrypLE Select and accutase may be used at a dilution of 1:1 with 0.02 % EDTA/PBS.
7. Add the culture medium.
8. Pipette the cells in the medium to disperse the cells completely (Fig. 1d). Transfer the dissociated cells to a 15-mL conical centrifuge tube.
9. After centrifugation at $200 \times g$ for 3 min at 4 °C, aspirate the supernatant. Resuspend the cells in medium, count the cells,
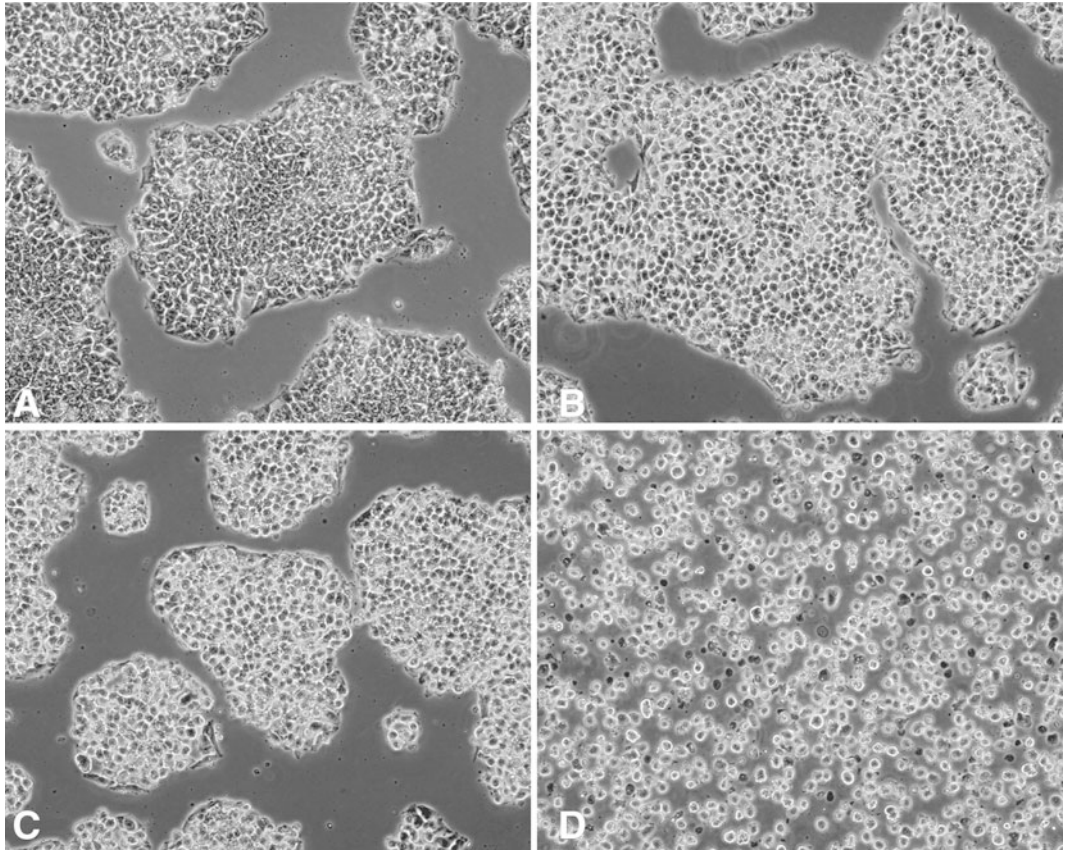
Fig. 1 (**a**) Appearance of KhES-1 colonies after EDTA treatment. (**b**, **c**) Appearance of hPSC colonies after (**b**) TrypLE Select or (**c**) accutase treatment. (**d**) hPSCs after single-cell dissociation. Bar indicates 50 μm

and re-seed at 4–6×10^4 cells/cm^2 or an appropriate density in a new culture vessel precoated with Synthemax. Add 10 μM ROCK inhibitor (e.g., Y-27632) to the culture medium. Ensure the cell suspension is eventually distributed in the wells by gently swirling the plate.

10. Starting at approximately 24 h post-seeding, change the medium daily. Use 3–4 mL medium/well for 6-well plates. In some case, changing the medium at 48 h post-seeding may be better. Subculture hPSCs every 4–5 days (Fig. 2).

3.2 Cryopreserving hPSCs

1. Prior to starting, equilibrate solutions to room temperature, except the cryopreservation medium.

2. Prepare cryopreservation medium. Place the tube containing freezing medium on ice until use.
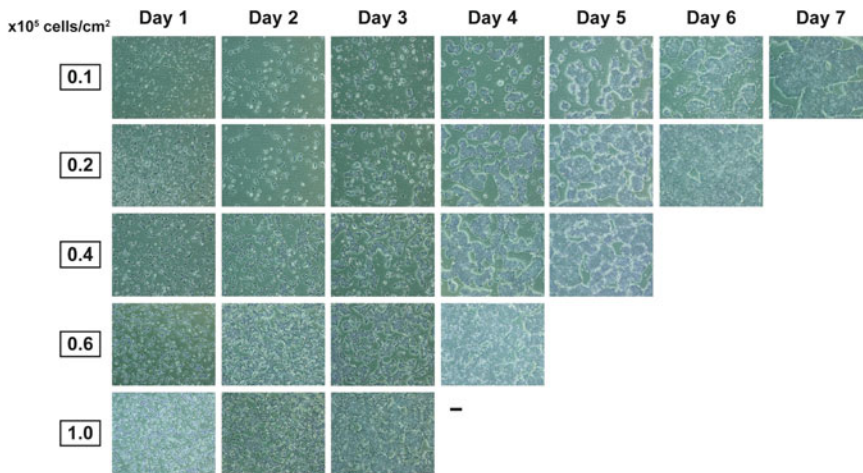
Fig. 2 Growth of KhES-3 cells cultured on Synthemax matrix with mTeSR1 medium at different cell densities. Bar indicates 100 μm

3. Prepare dissociated cells by passaging hPSCs. Centrifuge a 15-mL conical tube containing the cell suspension at $200 \times g$ for 3 min at 4 °C.

4. Aspirate the supernatant, resuspend in 2 mL culture medium, and count the cells.

5. Centrifuge the conical tube containing the cell suspension at $200 \times g$ for 3 min.

6. Aspirate the supernatant, resuspend in 0.5 mL culture medium, and count the cells.

7. With gentle agitation, add an equal volume of chilled cryopreservation medium dropwise. This technique reduces osmotic shock to the cells (*see* **Note 10**).

8. Place 1 mL of cell suspension in each freezing vial.

9. Transfer the cells to a freezing container and store at −80 °C overnight.

10. For long-term storage, transfer the cells to a liquid nitrogen tank with a vapor phase the following day.

3.3 Thawing hPSCs

1. Remove the vial of hPSCs from the liquid nitrogen storage tank.

2. Thaw the vial in a 37 °C water bath. Agitate the vial gently. Be careful not to completely submerge the vial under water.

3. Remove the vial from the water bath when only a small amount ice remains.

4. Spray the outside to the vial with 70 % ethanol.

5. Gently pipette the cells from the vial into a 15-mL conical centrifuge tube.
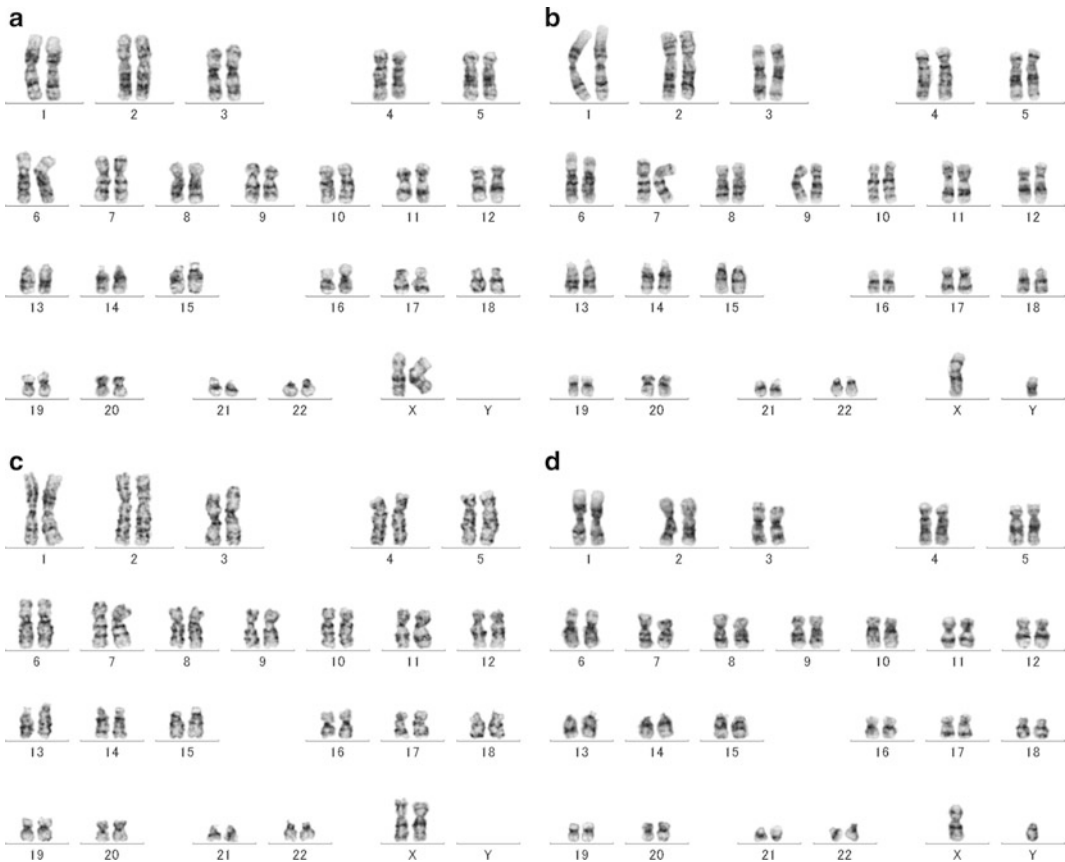
Fig. 3 Normal karyotypes are present in the hPSC lines under the experimental culture conditions. (**a**) KhES-1, (**b**) KhES-3, and (**c**) H9 hPSC lines cultured with TeSR2 medium over 20 passages. (**d**) KhES-3 hPSC line cultured with mTeSR1 medium over 20 passages

6. Slowly add 9.5 mL of complete culture medium dropwise to the cells while gently agitating the tube. This technique reduces osmotic shock to the cells (*see* **Note 10**).

7. Centrifuge the conical tube containing the cell suspension at $200 \times g$ for 3 min.

8. Aspirate the supernatant, resuspend the cells in 3–4 mL culture medium, and then count the cells.

3.4 Evaluation of hPSCs

We recommend monitoring the cultured cells periodically (every 5–10 passages) to ensure maintenance of pluripotency and a normal karyotype. We perform flow cytometric analyses to monitor several cell surface markers and transcription factors that are expressed in undifferentiated hPSCs. We recommend checking the karyotype by G-banding analysis as indicated in Fig. 3. Our recent results are presented in Table 1, which indicate that our protocol enables efficient expansion of hPSCs under feeder-free conditions with a non-biological substrate and defined/xeno-free medium.

Table 1

Flow cytometric analysis of hPSC lines. Markers of the undifferentiated state (OCT4, NANOG, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81) and a differentiation marker (SSEA-1)

Exp. No.	1				2				3				4		5	
Cell lines	KhES-3	KhES-3	KhES-3	KhES-3	H9	H9	H9	H9	KhES-1	KhES-1	KhES-1	KhES-1	KhES-3	KhES-3	KhES-1	KhES-1
Dissociation type Colony/Single	Colony	Colony	Single	Colony	Single	Colony	Colony	Single	Colony	Colony	Single	Colony	Colony	Single	Colony	Single
Media	DMEM/ KSR	PSGro	PSGro	NutriStem	NutriStem	DMEM/ KSR	TeSR2	TeSR2	DMEM/ KSR	TeSR2	TeSR2	NutriStem	DMEM/ KSR	TeSR2	DMEM/ KSR	TeSR2
No. of Passage	20	20	20	20	20	20	20	20	10	10	10	10	22	22	20	20
NANOG (+) %	97.7	92.2	99.6	96.2	98.3	99.2	99.7	99	99.1	98.9	99.6	95.3	99.2	99.4	98.9	99.3
OCT4 (+) %	97.9	92.3	98.9	95.6	99	98.7	99.4	98.5	99.1	98.9	99.5	95.5	97.5	99.5	98.9	99
SSEA1 (−) %	99.3	96.8	99.2	98	99.2	99.4	99.4	99.1	99.7	99.5	99.8	98.9	99.5	96.5	98.5	99.7
SSEA3 (+) %	95	83.6	41.3	84.8	85.8	96.9	82.8	96.3	93.3	89.7	93.8	91.1	89.6	95	95.4	96.9
TRA1-81 (+) %	96.4	78.5	99.4	94.5	87.9	85.2	80.2	85.7	98.2	96.7	98.3	97.1	88	97.9	98.3	97.4
TRA1-60 (+) %	95.9	88.9	99.4	96.2	95.3	60.6	84.1	87.2	97.2	94.1	98.6	96.1	89.1	ND	ND	ND
SSEA4 (+) %	100	100	100	99.9	100	100	100	100	100	100	100	99.9	100	ND	ND	ND

4   Notes

1. We strongly recommend mTeSR1 medium for initial experiments as defined, xeno-free medium.

2. There are often lot-to-lot variations among xeno-free media. Therefore, we recommend using the same lot of medium. We did not test other commercial xeno-free media such as Essential 8 (Invitrogen, cat. no. 1517001) or TeSR-E8 (STEMCELL Technologies, cat. no. ST-05940). Essential 8 and TeSR-E8 are low protein media, and they appear to be sensitive to enzymatic dissociation of hPSCs.

3. Synthemax II-SC substrate (Corning, cat. no. 3781) is more convenient because it can coat any vessel size according to the manufacturer's instructions. For comparison between LM-E8s and Synthemax, LM-E8s can be purchased from Nippi (iMatrix-511, cat. no. #892001, Tokyo, Japan).

4. For efficient cell dissociation, 0.02 % EDTA/PBS generally requires 5–10 min incubation.

5. Accutase is milder than TrypLE Select as a cell dissociation agent.

6. Prepare small aliquots (stock solution, ×1,000) at 10 mM in cell culture-grade water. Store at −20 °C or −80 °C. Stock solutions may be stored at 4 °C, but the activity will decrease.

7. Addition of Y-27632 to cryopreservation medium is generally unnecessary. However, Y-27632 may be effective for long-term handling of a large amount of cells.

8. Although the Human Pluripotent Stem Cell Transcription Factor Analysis Kit contains an anti-SOX2 antibody, we do not use this antibody because it often has problems. We do not use the anti-OCT4 antibody in the Human and Mouse Pluripotent Stem Cell Analysis Kit because there is an anti-OCT4 antibody in the Human Pluripotent Stem Cell Transcription Factor Analysis Kit. Similarly, we do not use the anti-SSEA-1 antibody in the Human and Mouse Pluripotent Stem Cell Analysis Kit because an anti-SSEA-1 antibody is included in the Human Pluripotent Stem Cell Sorting and Analysis Kit. Furthermore, we use a PE-conjugated anti-TRA-1-60 antibody with the Human and Mouse Pluripotent Stem Cell Analysis Kit.

9. Traditional hPSC culture methods require the use of mouse or human fibroblast feeder layers with Knockout Serum Replacement medium (Invitrogen, cat. no. 10828). Therefore, hPSCs need to adapt to new culture media and substrates. Most commercial media do not require an adaptation process. For some hPSC lines, better results may be obtained by gradually adapting to the defined/xeno-free medium on Matrigel as an intermediary step.

Alternatively, 50 % conditioned medium from mouse embryonic fibroblasts + 50 % defined/xeno-free medium on Matrigel can be used as a first intermediary step, following by culture in defined/xeno-free medium on Matrigel as a second intermediary step. Removing feeder cells is not essential because feeder cells are removed during passaging. We recommend split ratios of 1:2–1:5 for the first several passages of adaptation. Normally, a ratio of 1:10 is appropriate when the cells are fully adapted. As shown in Fig. 2, low-density culture is applicable, but we did not evaluate the cultured cells for an extended time.

10. For freezing and thawing hPSCs, reducing the osmotic shock is very important. By skipping this process, the viability of the cells will be very low.

Acknowledgments

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Microarray Approach to Identify the Signaling Network Responsible for Self-Renewal of Human Embryonic Stem Cells

Noboru Sato and Ali Brivanlou

Abstract

Here we introduce the representative method to culture HESCs under the feeder and feeder-free conditions, the former of which is used to maintain or expand undifferentiated HESCs, and the latter can be used for the preparation of pure HESCs RNA samples, or for screening factors influential on self-renewal of HESCs. We also describe a protocol and tips for conducting gene chip analysis focusing on widely used Affymetrix Microarrays. These techniques will provide us unprecedented scale of biological information that would illuminate a key to decipher complex signaling networks controlling pluripotency.

Keywords: Human pluripotent stem cells, Human embryonic stem cells, Human-induced pluripotent stem cells, Microarray, Pluripotency, Self-renewal, Mouse embryonic stem cells, Signaling network

1 Introduction

Stem cells can be defined by their two essential biological functions, namely eternal self-renew and generation of differentiated cell types (1, 2). Embryonic stem cells are pluripotent stem cells that have been derived from the inner cell mass (ICM) of blastocysts (1, 3, 4). It is unarguable that their differentiation potential is exceptionally versatile as compared to other stem cell groups in that they can give rise to all three germ-layer derivatives and germ cells (1). Mouse embryonic stem cells (MESCs) have been extensively used for generating mutant mice as a powerful reverse-genetics approach to identify in vivo biological functions of genes of interest (3–6). MESCs can be also utilized to understand molecular mechanisms that regulate early organogenesis by in vitro assay systems (7). The derivation of human embryonic stem cells (HESCs) (8, 9) has made enormous impact on stem cell research as they could be a possible source for tissue replacement therapy to cure critical diseases such as Parkinson's disease, diabetes mellitus, and cardiac infarction. The recent innovation of the induced pluripotent stem (iPS) cell technology that converts differentiated somatic cells into pluripotent stem cells now allows us to generate human pluripotent stem cells

without any ethical concerns (10–12). Despite their unique potential in understanding early human embryogenesis and generating human differentiated tissues, little is known about molecular mechanisms that govern HESCs identity.

There are a group of genes including Oct-3/4, Rex-1, and Nanog that are known to be specifically regulated in pluripotent MESCs (7, 13–19). Although it is likely that most of the essential molecular mechanisms underlying pluripotency are conserved between mouse and human (1, 8, 20), accumulating evidences suggest that there are unambiguous biological differences between MESCs and HESCs. The LIF/Stat3 pathway is sufficient to maintain MESCs in the undifferentiated state while this pathway does not support self-renewal of HESCs (1, 8). Trophoblasts can be derived from HESCs through activation of the BMP signaling pathway; whereas, MESCs are never differentiated into this lineage unless a specific gene function is disrupted (21, 22). Moreover, activation of the BMP signaling pathway can support self-renewal of MESCs in a certain culture condition rather than induction of differentiation (23). To better understand the molecular identity that determines self-renewing HESCs, we have conducted large-scale transcriptional profiling of HESCs using Affymetrix Gene chips. We found that approximately 900 genes are significantly enriched in the undifferentiated state in HESCs (24). Importantly, several molecular components integrated in major signal transduction pathways are specifically enriched in self-renewing HESCs indicating possible roles of specific signaling pathways in maintenance of pluripotency. Moreover, when compared to the reported MESCs-enriched genes, roughly 200 genes are overlapped between HESCs and MESCs, suggesting that these common genes may be involved in core molecular networks regulating pluripotency conserved between the two species. Here we introduce the representative method to culture HESCs under the feeder and feeder-free conditions, the former of which is used to maintain or expand undifferentiated HESCs, and the latter can be used for the preparation of pure HESCs RNA samples, or for screening factors influential on self-renewal of HESCs. We also describe a protocol and tips for conducting gene chip analysis focusing on widely used Affymetrix Microarrays. These techniques will provide us with unprecedented scale of biological information that would illuminate a key to decipher complex signaling networks controlling pluripotency.

2   Materials

2.1   Tissue Culture

1. mTeSR1 (StemCell Technologies, #05850).
2. mFreSR (StemCell Technologies, #05855).
3. DMEM/F-12 (Gibco, #11320-033).

4. DMEM with D-glucose and L-glutamine, and pyridoxine hydrochloride, but no sodium pyruvate nor sodium bicarbonate (Gibco, #12100-046).

5. Dulbecco's Phosphate-Buffered Saline (1×) without calcium/magnesium (Gibco, #14190-250).

6. Knockout-Dulecco's modified Eagle's medium (Gibco, #10829-018).

7. Knockout Serum Replacement (Gibco, #10828-028).

8. Fetal Bovine Serum (HyClone, #SH30070.03).

9. 2.0 mM L-Glutamine (Gibco, #25030-081).

10. 0.1 mM Nonessential Amino Acids (Gibco, #11140-050).

11. Human Recombinant Basic Fibroblast Growth Factor (Gibco, #13256-029).

12. 0.05 % Trypsin-EDTA (Gibco, #25300-054).

13. Dispase, lyophilized (Gibco, #17105-041).

14. Cell Dissociation Buffer (Sigma, #C5914).

15. 55 mM (1,000×) 2-Mercaptoethanol (Gibco, #21985-023).

16. Penicillin/Streptomycin (Gibco, #15070-063).

17. Gelatin, TypeA (Sigma, #G-1890).

18. Matrigel (BD Biosciences, #354234).

19. NYL Filter Unit, 500 ml (Nalgene, #151-4020).

20. 0.2 μm syringe filter (Gelman, #4192).

21. Multiwell 6-well Tissue Culture-Treated plate (FALCON, #353046).

22. 10 × 20 mm Cell Culture Dish (Corning, #430167).

23. 14 ml Polypropylene Round Bottom Tube (FALCON, #352059).

24. 50 ml Polypropylene Conical Tube (FALCON, #352098).

2.1.1 Human Embryonic Stem Cell Lines

There are 279 HESCs lines currently eligible for federal funding of HESCs research (*see* **Note 1**). We have been mainly using WA01 (H1) and WA09 (H9) (WiCell Research Institute) for most of the experiments because there were only few HESCs lines available at the time we started HESCs research, and suitable for the feeder-free culture system. We therefore focus on introducing the culture method for these HESCs lines.

2.1.2 Mouse Embryonic Fibroblasts (MEFs)

A large stock of MEFs can be prepared from E13 or E14 mouse embryos according to the generally used protocol. MEFs must be mitotically inactivated by irradiation or mitomycin C treatment before using for feeder cells (*see* **Note 2**).

2.1.3 Medium for Human Embryonic Stem Cells (HESCs)	For H1 and H9 cell lines (WiCell Research Institute), we routinely use mTeSR^{TM}1 (StemCell Technologies) for the feeder-free culture condition. The same company also provides the more defined version without animal-derived components, TeSR^{TM}2, for preclinical research. Alternatively, we occasionally use DMEM/ F-12 supplemented with 20 % KSR, 1.0 mM L-glutamine, 0.1 mM non-Essential Amino Acids, 50 U/ml penicillin/streptomycin (*see* **Note 3**), 0.1 mM 2-Mercaptoethanol, and 4 ng/ml of basic FGF for feeder-free and feeder-dependent culture conditions.

2.1.4   Medium for Mouse Embryonic Fibroblasts (MEFs)

We use DMEM supplemented with 10 % FBS, and 50 U/ml of penicillin/streptomycin for cultivation of MEFs.

2.1.5   Dispase Solution

We use Dispase for the passaging of H1 and H9 cells. Alternatively, Collagenase can be also used as WiCell recommends in their protocol (*see* **Note 4**).

1. Weigh appropriate amount of Dispase and dissolve in HESCs (H1) medium to make a final concentration of 6 U/ml.

2. If necessary, warm up the solution in a 37 °C water bath to fully dissolve Dispase.

3. Filter the Dispase solution through 0.2 μm syringe filter (*see* **Note 5**).

2.1.6   Equipments for Embryonic Stem Cell Culture

We use regular cell culture equipments for HESCs and MESCs culture.

We separate CO_2 incubators for the maintenance of HESCs, MESCs, or other general cell cultures. All procedures should be performed by following the general sterile tissue culture techniques (*see* **Note 6**).

1. Tissue culture hood.

2. CO_2 incubator.

3. 37 °C water bath.

4. −70 °C freezer.

5. −20 °C freezer.

6. 4 °C refrigerator.

7. Liquid nitrogen tank.

8. Centrifuge.

9. Inverted microscope.

10. Freezing container.

2.2 GeneChip

There are many different types of high throughput gene array systems. We use Affymetrix micoarrays because of their highly organized and reliable basic structures and interchangeable flexible format. Most of the reagents are available from Affymetrix (*see* **Note 7**). Recently, RNA-seq technologies have emerged that offer several advantages over the microarray analysis such as detection and quantification of novel splicing isoforms and exons and the higher sensitivity for genes expressed at quite low levels. There are however still considerable benefits found in the gene chip analysis. For instance, since the cost for each gene chip experiment is less expensive than that for RNA-seq, it is advantageous when a large group of samples need to be analyzed. In addition, due to the continuous improvement of analytical strategies accumulated in the past decade, the updated data processing and mining approaches for gene chip analysis already take account for the potential drawbacks and biases generated from the gene chip experiments.

1. GeneChip® WT PLUS Reagent Kit (Affymetrix, #902280)

   (a) WT Amplification Kit Module 1

   First-Strand Enzyme, First-Strand Buffer, Second-Strand Enzyme, Second-Strand Buffer, IVT Enzyme, IVT Buffer, Control RNA (1 mg/ml HeLa total RNA), 2nd-Cycle Primers, 2nd-Cycle ss-cDNA Enzyme, 2nd-Cycle ss-cDNA Buffer, RNase H, and Nuclease-free Water

   (b) WT Amplification Kit Module 2

   Purification Beads

   (c) GeneChip® Poly-A RNA Control Kit

   Poly-A Control Stock and Poly-A Control Dil Buffer

   (d) GeneChip® WT Terminal Labeling Kit

   $10\times$ cDNA Fragmentation Buffer; UDG, 10 U/μL; APE 1, 1,000 U/μL; $5\times$ TdT Buffer; TdT, 30 U/μL; DNA Labeling Reagent, 5 mM; and RNase-free Water

   (e) GeneChip® Expression 3′-Amplification Reagents Hybridization Control Kit, $20\times$ Hybridization Controls and 3 nM Control Oligo

2. GeneChip® Fluidics Station 450 (Affymetrix, #00-0079)

3. GeneChip® Scanner 3000 7G (Affymetrix, #00-0212)

4. GeneChip® AutoLoader with External Barcode Reader (Affymetrix, #00-0090 (GCS 3000 7G S/N 501) #00-0129 (GCS 3000 7G S/N 502))

5. GeneChip® Hybridization, Wash, and Stain Kit (Affymetrix, #900720)

6. Affymetrix® GeneChip® Command Console® 4.0 (#702569)

7. Affymetrix® Expression Console™ Software

3 Methods

3.1 HESCs Culture

HESCs can be grown on either conventional MEFs feeder cells or Matrigel under the feeder-free condition. When compared to HESCs grown on MEFs feeder cells, HESCs may not be easily kept undifferentiated under the feeder-free condition as several factors including the condition of Matrigel coating and MEFs-conditioned medium would substantially affect self-renewal of HESCs. We therefore recommend that a primary batch of HESCs (in a cryovial tube sent from the provider) should be grown on MEFs feeder cells during initial several passages until a large enough number of frozen stocks in lower passages is prepared (*see* **Note 8**).

3.1.1 Cultivation of MEFs as Feeder Cells for HESCs

1. Thaw a MMC-treated frozen vial of MEFs in a 37 °C water bath by gently swirling.

2. Plate cells on 0.1 % Gelatin-coated dishes at 2×10^4 cells/cm^2 in MEFs medium.

3. 24 h later, rinse MEFs with HESCs medium once to remove residual MEFs medium prior to plating HESCs.

3.1.2 Cultivation of HESCs on MEFs Feeder Cells

We recommend that a researcher who works on HESCs for the first time may start growing HESCs on MEFs-feeder cells rather than using the feeder-free system because of the complexity in the latter method as mentioned above. In addition, it is beneficial to understand the morphology of undifferentiated HESCs when perfectly maintained on MEFs so that the subtle sign of differentiation can be immediately noticed when HESCs are grown under the feeder-free condition. Having experience in growing MESCs is beneficial but not absolutely required, since the maintenance of HESCs is far quirkier than that of MESCs. Special attention must be paid at each process, and careful daily observation of HESCs through the inverted microscope is essential to get used to maintaining HESCs in the best condition.

1. Thaw a frozen vial of HESCs in a 37 °C water bath by gentle agitation before the last trace of ice melts (*see* **Note 9**).

2. Wipe the outside of the vial with 70 % Ethanol and immediately transfer the content to a 15-ml of conical tube filled with 10 ml of HESCs medium.

3. Gently pipette the cells up and down no more than five times.

4. Centrifuge the collected cells at $200 \times g$ for 5 min.

5. Aspirate the supernatant, and gently flick the bottom of the centrifuge tube to dissociate the cell pellet.

6. Add an appropriate amount of HESCs medium (feeder-layer culture) or CM (feeder-free culture) and resuspend the cells by gently pipetting.

7. Plate HESCs into culture vessels at the optimal concentration.

3.1.3 Passaging

We passage HESCs at between 3 and 5 days after initiation of culture before they start reaching confluency or showing any signs of differentiation.

1. Aspirate culture medium.

2. Add 1 ml of Dispase solution or Cell Dissociation Buffer to HESCs in one well of a 6-well plate.

3. Incubate the culture plate at 37 °C (incubator) for 5–10 min (Dispase) or 3–5 min (Cell Dissociation Buffer) (*see* **Note 10**).

4. Carefully observe HESCs under the inverted microscope periodically to see if they start detaching from the surface.

5. Remove HESCs from the culture surface by pipetting them using P1000 pipetman (*see* **Note 11**) or scraping them using 5 ml plastic or glass pipette (*see* **Note 12**).

6. Transfer HESCs into HESCs medium (more than six times the volume of Dispase solution used at step 2. e.g., 18 ml of HESCs medium for 3 ml of Dipase solution) and briefly pipette them to fully dilute the Dispase solution.

7. Centrifuge at 1,000 rpm for 5 min.

8. Aspirate supernatant.

9. Briefly tap the bottom of the tube to gently dissociate HESCs pellet.

10. Resuspend HESCs in appropriate amount of HESCs medium (e.g., 2–2.5 ml for one well of a 6-well plate).

11. Aspirate HESCs medium from MEFs-coated plates.

12. Plate HESCs onto MEFs-coated plates.

3.1.4 Freezing HESCs

When HESCs reach subconfluency in the culture plates, cells can be harvested and stored at −80 °C.

1. Harvest HESCs according to the protocol shown in the passaging section.

2. After washing cells with HESCs medium two times, resuspend the pellet with the freezing medium consisting of 10 % DMSO,

30 % FBS, and 60 % HESCs medium. Alternatively, HESCs can be conveniently preserved in the premade freezing medium, mFreSR (StemCell Technologies).

3. Add 1 ml of cell suspension to each cryovial tube.

4. Freeze cryovial tubes in a freezing container at −70 °C overnight.

5. The next day, place the frozen cryovial tubes in the liquid nitrogen tank.

3.1.5 Cultivation of HESCs in the Feeder-Free Condition

The feeder-free methods have provided a novel way to culture HESCs in the absence of direct contact with MEFs (25). In this system, HESCs are maintained in the undifferentiated state by growing on a Matrigel-coated plate in medium-conditioned from MEFs (CM). Recently, the system has been updated by the combination with the defined complete medium such as mTeSR1 which no longer requires MEF to make conditioned medium. This method is characterized by two major advantages as compared to the conventional MEFs-feeder method. First, this method provides us with pure, undifferentiated HESCs RNA or protein samples without any contaminated MEFs-derived materials. This is particularly critical when HESCs are subjected to Gene chip analysis because this method is highly sensitive, and potentially detects any contaminated RNA samples whose sequences are similar to ones in human. The second advantage is that since the undifferentiated state of HESCs largely relies on CM or the complete medium, this system allows us to identify a factor(s) involved in maintenance of self-renewal of HESCs. Under the feeder-free condition, HESCs change their morphology from tight and compact undifferentiated shape to the flattened differentiated shape as early as 1 day after switching from CM or complete medium to non-CM (*see* Fig. 1). Thus, soluble factors that support self-renewal of HESCs can be screened simply through the addition of candidate factors to non-CM in this system. Drawback of this system is however that several variable factors such as the quality of Matrigel and CM could substantially influence the undifferentiated condition of HESCs. Moreover, it has been reported that HESCs passaged under the feeder-free condition prone to be accompanied with chromosomal abnormality at relatively lower passages than that of HESCs grown on MEFs feeders (*see* **Note 13**) (26). It is therefore recommended to use this system for evaluation of undifferentiated HESCs in the experiment within several passages, rather than for preparation of master stocks or routine maintenance of HESCs. In case a longer passaging under the feeder-free condition is required, regular karyotyping is encouraged to ensure the quality of HESCs. An example of the culture plan of HESCs under the feeder-free condition is shown below (*see* **Note 14**).
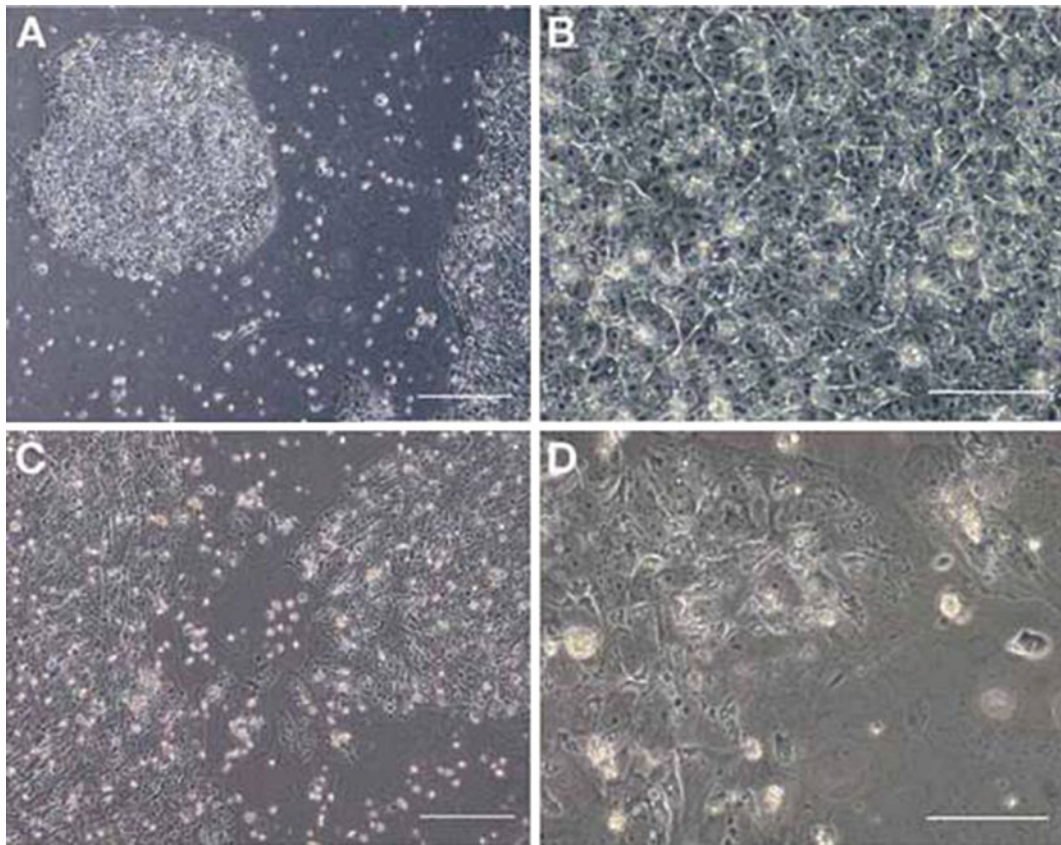
Fig. 1 Morphology of HESCs (H1 line) cultured under the feeder-free condition. HESCs were grown in conditioned medium (CM) (**a**, **b**) or non-CM (**c**, **d**) for 2 days under the feeder-free condition and photographed by phase-contrast microscopy (**a**, **c**: low magnification; **b**, **d**: high magnification). Note compact and tight cell morphology representative of the undifferentiated HESCs (**a**, **b**) grown in CM. HESCs grown in non-CM show flattened and larger cytoplasm typical for differentiating HESCs (**c**, **d**). Scale bars: (**a**, **c**) 100 μm; (**b**, **d**) 50 μm

1. Culture schedule when using CM.

   Day 1: Plate MEFs on gelatin-coated dishes. Thaw Matrigel on ice overnight.

   Day 2: Replace MEFs medium with HESCs medium. Dilute Matrigel with DMEM/F-12, coat dishes with diluted Matrigel, and leave them on ice overnight.

   Day 3: Collect CM from MEFs culture. Rinse Matrigel-coated plates with DMEM/F-12 once. Thaw a frozen vial of HESCs and plate them on Matrigel-coated plates along with CM.

2. Culture schedule when using the defined complete medium (e.g., mTeSR1).

Day 1: Dilute Matrigel with DMEM/F-12, coat dishes with diluted Matrigel, and leave them on ice overnight.

Day 2: Rinse Matrigel-coated plates with DMEM/F-12 once. Thaw a frozen vial of HESCs and plate them on Matrigel-coated plates along with the defined complete medium.

3.1.6 Preparation of Conditioned Medium (CM) from MEFs

We culture MEFs for up to 7–8 days for preparation of CM. Beyond this period, activity of CM seems to be decreased as judged by the ability to keep HESCs undifferentiated. It could be however variable depending on the batch and cell density of MEFs plated on the dish.

1. Coat 6-well plates or 10 cm dishes with 0.1 % Gelatin for more than 20 min followed by rinsing with PBS two times (*see* **Note 15**).

2. Plate MMC-treated MEFs on the gelatin-coated dishes at 1×10^5 cells/cm^2 in MEFs medium.

3. After overnight incubation, rinse MEFs with HESCs medium once (8 ml/dish) to remove residual MEFs medium and feed MEFs with 10–12 ml of HESCs medium.

4. 24 h later, recover the medium to use as MEF-conditioned medium (CM) (*see* **Note 16**).

5. Add additional 4 ng/ml of bFGF to CM prior to feeding HESCs.

3.1.7 Preparation of Matrigel-Coated Plates

This step is one of the most critical steps in preparation of the feeder-free culture of HESCs. As the condition of the coated-Matrigel significantly influences self-renewal of HESCs, one might need certain experience in growing HESCs on MEFs feeder cells to recognize if HESCs are in the good (undifferentiated) condition or not.

1. Thaw one vial (10 ml) of Matrigel stored at −20 °C on ice overnight.

2. Aliquot 1 ml of Matrigel in each cryovial tube and store at −20 °C (*see* **Note 17**).

3. One day before preparation of plates, thaw the frozen Matrigel aliquote on ice overnight.

4. Suspend 1 ml of Matrigel in 20 ml of DMEM/F12 and plate on 6-well plates at 1 ml per well (*see* **Note 18**).

5. Keep the Matrigel-coated plates on ice overnight while it is horizontally positioned so that each well is fully covered with Matrigel.

6. Prior to initiation of HESCs culture, incubate Matrigel-coated plates in the CO_2 incubator for more than 30 min to allow them to gelatinize.

7. Rinse the Matrigel-coated plates with DMEM/F-12 once to remove excessive Matrigel in each well (*see* **Note 19**).

3.1.8 Passaging of HESCs in the Feeder-Free Condition

We passage HESCs at around 3–5 days after initiation of culture in the feeder-free condition. Beyond this period, HESCs at the margin or center of the colony start changing their morphology to form a flat differentiated shape.

All the passaging procedures are the same as that of HESCs grown on MEFs-feeder cells with exceptions of using CM or defined complete medium instead of HESCs medium when resuspending HESCs prior to plating, and using Matrigel-coated plates instead of MEFs-coated plates when plating HESCs. Importantly, when evaluating a factor(s) involved in the undifferentiated state in the screening process, it is beneficial to dissociate HESCs colonies into small aggregates since smaller colonies are more sensitive to the differentiation environment than larger ones, which are resistant to the differentiation signal. Although the viability of HESCs substantially falls when cells are fully dissociated, we and others have recently reported that the cell survival can be efficiently restored by using the inhibitors of Rho-associated kinase (Rock) or its downstream target, nonmuscle myosin II (27–30).

3.1.9 Preparation of Frozen Stocks of HESCs in the Feeder-Free Condition

HESCs grown under the feeder-free condition can be stored in cryovials in the same way that is used for HESCs grown on MEFs-feeder cells.

3.2 GeneChip

Microarray approach has become one of the most powerful molecular techniques to comprehensively illuminate multiple transcriptional networks regulating complex biological phenomenon. Although most of the experimental processes are highly organized and even automated in the Affymetrix microarray system, incompletion of any given step (e.g., insufficient mixture of reagents) would substantially influence the final data quality. It is therefore quite essential to rigorously inspect the source samples (e.g., the initial sample RNA, cRNA after IVT) without any compromise before proceeding to the next step. This should be further emphasized to avoid wasting time on data analysis step for the low quality raw data because in silico processes including data extraction, filtration, and interpretation are generally far more time-consuming and labor-intensive work than wet-experimental steps (*see* **Note 20**). If one of the triplicate samples has less quality than the others, the entire resolution of the data analysis could be severely impinged through statistical filtration resulting in exclusion of many actually

regulated genes. For these reasons, when the sample does not reach the highest standard, we repeat the experimental procedure until the sample quality meets the required level rather than proceeding to the next step.

3.2.1 Experimental Plan

Since the microarray project generally requires a considerable amount of time, effort, and budget, it is not to mention that the precise experimental planning is the primary key to succeed in the entire project. First, you need to determine how many conditions you want to compare (e.g., HESCs vs. adult-derived tissues, HESCs in the differentiation condition between different time points). Then, you can estimate the total number of arrays to be used by multiplying the number of conditions by the number of replicate per each condition. It should be noted that because array data is intrinsically accompanied with a certain level of signal variations between different samples even in the exact same condition, statistical filtration of the raw signal is inevitable to reliably select enriched genes while excluding potential noises. We therefore strongly recommend to set up at least triplicate samples in each condition.

3.2.2 Preparation of Total RNA from HESCs

Extract RNA from HESCs grown in the triplicate condition using Qiagen RNeasy kit or other equivalent RNA extraction kits. As mentioned above, since the successful gene array experiment largely relies on the quality of the starting materials, the purity of the sample RNA should not be compromised. We routinely evaluate the extracted RNA by UV spectrophotometer for protein contamination, and by the RNA Nano Lab chip for RNA degradation, and use only the highest quality of the RNA sample for the following procedure.

3.2.3 First-Strand cDNA Synthesis (Example: Total RNA Between 250.0 and 500.0 ng*)

Use 3′ IVT Amplification Kit Module 1 for this step.

(a) Mix 4 μL 3′ First-Strand Buffer and 1 μL of 3′ First-Strand Enzyme (total volume First-Strand Master Mix = 5 μL).

(b) Mix X μL (250.0–500.0 ng), total volume (5 μL) of the sample RNA, 5 μL First-Strand Master Mix and (5-X) μL of DEPC-H2O. Centrifuge the tube to spin down the condensation.

(c) Incubate at 42 °C for 2 h, and place on ice for at least 2 min (use a PCR machine or a cooling water bath).

3.2.4 Second Strand cDNA Synthesis

Use 3′ IVT Amplification Kit Module 1 for this step.

(a) Mix 5 μL 3′ Second-Strand Buffer, 2 μL of 3′ Second-Strand Enzyme and 13 μL RNase-free water (total volume Second-Strand Master Mix = 20 μL). Add the following materials to the First-Strand cDNA reaction.

(b) Add 10 μL of the sample first-strand cDNA to the 20 μL Second-Strand Master Mix.

(c) Mix the contents, and centrifuge the tube to spin down the condensation.

(d) Incubate at 16 °C for 1 h, then at 65 °C for 10 min, and place on ice for at least 2 min.

3.2.5 In Vitro Transcription (IVT)

Use 3′ IVT Amplification Kit Module 1 for this step.

(a) Mix 20 μL 3′ IVT Buffer, 6 μL of 3′ IVT Enzyme, and 4 μL of 3′ IVT Biotin Label (total volume IVT Master Mix = 30 μL).

(b) Add 30 μL of the sample Second-strand cDNA to the 30 μL IVT Master Mix.

(c) Mix the contents, and centrifuge the tube to spin down the condensation.

(d) Incubate at 40 °C for 4 h, and place on ice.

(e) The final reaction can be stored at –20 °C or subjected to the next step.

3.2.6 Cleanup of In Vitro Transcription

Use 3′ IVT Amplification Kit Module 2 for this step.

(a) Add 100 μL of Purification Beads to the in vitro transcription (IVT) reaction (60 μL) in a 1.5-ml tube and mix by pipetting.

(b) Incubate for 10 min to bind the cRNA to the beads.

(c) Collect the Purification Beads at the bottom of the tube with a magnet and discard sample supernatant.

(d) Wash the Purification Beads with 200 μL of 80 % ethanol for 30 s three times.

(e) Air-dry the Purification Beads to remove all the remaining liquid.

(f) Incubate for 1 min with 27 μL preheated (65 °C) RNase-free water to elute the cRNA.

(g) Transfer the aqueous phase to a fresh tube and place on ice.

(h) The final reaction can be stored at –20 °C or –70 °C or subjected to the next step.

3.2.7 Quantification of the IVT Sample (cRNA)

(a) Quantify the amount of total RNA in the IVT sample by using spectrophotometer.

(b) The quality of the RNA sample can be evaluated by RNA Nano Lab chip.

(c) Determine the cRNA yield by subtracting the amount of total RNA used for the First-Strand cDNA synthesis from the amount of RNA obtained after IVT as shown below.

Amount of RNA in the IVT sample (RNA_{IVT})

Amount of total RNA used for First-Strand cDNA synthesis (RNA_{First})

Amount of cDNA used for IVT/Amount of total Double-Stranded cDNA ($cDNA_{IVT/Total}$)

$$cRNA = RNA_{IVT} - RNA_{First} \times cDNA_{IVT/Total}$$

3.2.8 cRNA Fragmentation

Use 3′ IVT Amplification Kit Module 2 for this step.

(a) Take 15 μg of cRNA (≤32 μL) from the purified IVT sample and put it in a 1.5-ml tube.

(b) Add 8 μL of 3′ Fragmentation Buffer to the tube.

(c) Add RNase-free water to make a final volume of 40 μl.

(d) Incubate the reaction at 94 °C for 35 min, and place it on.

3.2.9 Hybridization of the Probe Array

Use GeneChip Hybridization Control Kit for this step.

(a) Prehybridize the Probe array cartridge with 200 μl (for the standard cartridge) of 1× Hybridization buffer through a septa backside of the array by using a P200 Pipetteman. Avoid air contamination that may result in uneven prehybridization of the array.

(b) Place the array cartridge in the hybridization oven at 45 °C for 10 min with rotations at 60 rpm.

(c) Mix the following reagents in a 1.5 ml tube.

3.7 μL Control oligo B2 (3 nM)

11 μL 20× Hybridization Controls (bioB, bioC, bioD, cre)

110 μL 2× hybridization mix

22 μL DMSO

43.9 μL RNase-free water

(total volume Hybridization Master Mix = 190.6 μL)

(d) Mix 29.4 μL (11 μg) fragmented and labeled cRNA and 190.6 μL of Hybridization Master Mix (total volume Hybridization Cocktail = 220 μL).

(e) Boil the hybridization mixture at 99 °C for 5 min followed by incubation at 45 °C for 5 min.

(f) Centrifuge the hybridization cocktail briefly and collect the aqueous phase.

(g) Remove the 1× Hybridization buffer used for prehybridization from the array, and fill up with the hybridization cocktail while avoiding contamination of insoluble materials at the bottom of the tube.

(h) Incubate the Probe array in the hybridization oven at 45 °C for 16 h with rotation at 60 rpm.

3.2.10 Array Data Analysis

(a) After 16 h of hybridization, the Probe array is washed, stained, and scanned to extract data from the array. The raw data is analyzed by using Expression Console™ Software.

(b) To estimate the statistical significance of differential gene expression between two groups (e.g., HESCs in the undifferentiated state vs. HESCs in the differentiated state) of three replicates each, we used a regularized t-statistics as described by Long and Baldi (31). All Signal values were first log-transformed and the regularized variances were taken as $\sigma_{reg}^2 = \frac{v_0\sigma_0^2 + (n-1)s^2}{v_0 + n - 2}$, independently for each group of replicates. We used $v_0 = 3$, s^2 as the sample variance ($n = 3$). σ_0^2 denotes the a priori expected variance for a gene, given its mean intensity, and is estimated from the data using an intensity-dependent regression of sample variance vs. sample mean. The means and variances from both groups were used in the same regression.

(c) For comparison of genes between different species (e.g., HESCs and MESCs), use NetAffx (www.netaffx.com) and TIGR database to search orthologous genes. It should be noted however that a certain number of genes in one species may not be able to correlate to the ortholog of other species due to still ongoing database construction.

(d) Once a group of genes that are distinctively expressed between two conditions are determined, the genes can be classified to subgroups by focusing on specific categories (e.g., gene function; Gene Ontology, protein domain, signaling pathways). In general, multiple different probes in the Affymetrix Gene Array can be corresponding to the same single gene. To determine how many distinct genes are included in the selected group, use NetAffx database to find out Unigene ID corresponding to each Probe ID so that overlapping genes are easily excluded. Validity of the selected group genes can be confirmed by evaluating whether known differentially regulated genes between the two conditions (e.g., Oct-3/4 and Nanog in the undifferentiated state) are enriched in the selected gene pool (*see* **Note 21**) (24).

4 Notes

1. To know more information regarding HESCs lines in the NIH Human Embryonic Stem Cell Registry, visit http://grants.nih.gov/stem_cells/registry/current.htm.

2. Frozen vials of mitomycin C-treated or untreated MEFs can be available from several companies (e.g., EMD Millipore).

3. The original recipe for H1 medium from the provider (WiCell Research Institute) does not contain any antibiotics. We however found that the addition of penicillin/streptomycin to HESCs medium does not substantially affect any character of self-renewing H1 cells, and even beneficial to avoid risk of bacterial contamination. Since penicillin/streptomycin has no effect on fungi or mycoplasma, careful sterile culture procedure is always critical for each culture step.

4. Add Collagenase to HESCs medium to make a final concentration of 1 mg/ml and dissolve in a 37 °C water bath. Filter the Collagenase solution using 0.2 µm syringe filter.

5. It is recommended to prepare fresh Dispase solution for each passaging procedure. Alternatively, the solution can be kept at 4 °C for several days.

6. It is quite important that the operator must wear gloves and glasses during cell culture procedures to prevent any possible cell culture–related troubles (e.g., virus infection, explosion of a frozen vial upon thawing).

7. Although we used Human Genome U133A and U133B chips that were separated, a combined gene array, Human Genome U133 2.0 Plus that covers entire human genome in one array is currently available.

8. It is of note that since chromosomal abnormality is reported to be occasionally found in high passaged HESCs in either culture method, regular karyotyping is recommended to ensure the condition of HESCs especially at higher passages (26).

9. It is particularly important to thaw the frozen vial as quick as possible to minimize damage to the cells. Usually it is completed within 1–2 min.

10. We consider 10 min as a maximum incubation period for Dispase because beyond this time, viability and quality (undifferentiated state) of HESCs after passaging is steeply decreased.

11. Although HESCs sometimes tightly adhere to the culture surface and are refractory to detach from the surface, the pipetting procedure should be completed in a shortest period to minimize the time in which HESCs are exposed to Dispase solution. If necessary, we even leave a part of HESCs colony unharvested to prioritize quality over quantity.

12. It is usually recommended that HESCs colonies should be kept as large as possible when being passaged to maintain their viability and undifferentiated state.

13. Although the exact reason is unclear, it could be due to selected growth of certain cell populations that acquired growth advantage through the chromosomal rearrangement under relatively higher selective pressure driven by the feeder-free condition.

14. It usually takes two to three passages until HESCs become free from contaminated MEFs in the feeder-free condition.

15. The Gelatin solution supports the attachment of MEFs on culture vessels. This treatment is especially important when MEFs are fed with HESCs (for H1) medium that induces a substantial morphological change in MEFs.

16. CM can be used immediately after collection or can be stored at $-80\ ^{\circ}C$ for several weeks.

17. Cryotubes should be prefrozen at $-20\ ^{\circ}C$ before making aliquotes to avoid gelation of Matrigel. Pipetting and aliquoting procedures should be done as quickly as possible to keep Matrigel cold.

18. Property of Matrigel appears to be slightly different from batch to batch. It is therefore quite important to predetermine the optimal concentration of Matrigel for each batch.

19. Optimally coated Matrigel demonstrates a well-formed mesh-like structure under the inverted light microscope. When the coated Matrigel is too diluted, the mesh-like structure is less formed, resulting in flattening and differentiation of HESCs on the Matrigel even in the presence of CM. If the coated Matrigel is too dense, Matrigel tends to remain aggregated even after rinsing with DMEM/F-12, and prevents HESCs from attaching and spreading on the surface.

20. There are several ways to computationally adjust the obtained data to fit representative profiling. There is however no way to make up for fundamental blemish at the raw data level.

21. It is also strongly recommended to examine expression of the selected genes by RT-PCR to confirm differential transcriptional regulation of the enriched genes between the two conditions.

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Monitoring Stemness in Long-Term hESC Cultures by Real-Time PCR

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Abstract

Human embryonic stem cells (hESC) involve long-term cultures that must remain undifferentiated. The real-time PCR (RT-PCR) technique allows the relative quantification of target genes, including undifferentiation and differentiation markers when referred to a housekeeping control with the addition of a calibrator that serves as an internal control to compare different lots of reactions during the time. The main aspects will include a minimal number of cells to be analyzed, genes to be tested, and how to choose the appropriate calibrator sample and the reference gene. In this chapter, we present how to apply the RT-PCR technique, protocols for its performance, experimental setup, and software analysis, as of the gene expression of hESC lines in consecutive passages for long-term culture surveillance.

Keywords: Real-time PCR, Relative quantification, hESC, Monitoring stemness

1 Introduction

The main objective of human embryonic stem cells (hESCs) is their future application in regenerative medicine (1–5). For that purpose, the main consideration to be taken into account is to establish standardized conditions to keep them in long-term cultures in an undifferentiated stage. Nowadays, most laboratories involved in hESC culture and maintenance have developed different systems to check stemness, although no real consensus exists regarding which markers should be used for routine survey. Therefore, it is important to establish standard quantitative monitoring systems that can alert of unwanted differentiation within our hESC cultures.

Over recent years, real-time PCR (RT-PCR) has become essential for nucleic acid quantification because it has unsurpassed sensitivity and a broad dynamic range. Before RT-PCR was available, conventional PCR methods based on end-point analysis were used for quantification of a specific target. Such methods have well-known limitations and disadvantages, i.e., reactions with a low initial copy number that can reach the same plateau as reactions starting with higher template concentrations and/or a different PCR efficiency. In contrast, RT-PCR provides accurate, kinetic quantification because it allows data analysis in the only phase

(log-linear phase) where the amplification efficiency of each reaction is constant, as assessed by fluorescent emission (due to the incorporation of SYBR Green).

This chapter describes the use of RT-PCR to monitor seven genes involving undifferentiation and differentiation markers referring to a housekeeping gene as well as the selection of a proper calibrator. This monitoring approach involves the quantitative gene expression surveillance of typical gene markers of undifferentiation *OCT3/4* (*POU5F1*), *NANOG*, and *REX1* (*ZFP42*) ([6–8]). On the other hand, unwanted differentiation must also be discarded, thus rendering necessary the selection of early differentiation of ectoderm, mesoderm, and endoderm represented by keratin (*KER*), cartilage matrix protein (*CMP*), amylase (*AMY*), and α-fetoprotein (*AFP*), respectively ([9]). This monitoring system can be applied to not only compare different hESC lines but also to survey a specific hESC line in long-term cultures. Although the LightCycler® (Roche) instrument is specified in this unit, the protocols may be utilized for any RT-PCR instrument.

The LightCycler® (Roche) system provides various experimental approaches to quantitative PCR, involving absolute and relative quantification. The most powerful is relative quantification, because the absolute values for most gene expression studies of the sample under investigation are not relevant. Various techniques express the target amount of an unknown sample in relation to another gene transcript, the so-called housekeeping gene, which is assumed to be constant. That identical concept can be achieved with the LightCycler® which determines target gene levels in relation to the levels of a reference gene in the same sample (see Table 1).

Furthermore, if calibrator normalization is applied to all the result lots, two additional benefits can be derived: PCR efficiency correction, achieved by standard curve construction, and the comparison of the results in the different runs performed, i.e., to track changes over time. To correct PCR efficiency, standard curves are constructed for all the genes of interest from which RNA (cDNA) is measured, and linear regression analysis is applied to interpolate unknown sample values. The standard curve assay may be performed even if the PCR amplification efficiencies of the primer sets are not equal since the correction for unequal efficiencies is intrinsic to the linear regression formula.

2 Materials

2.1 Collection of Cells

1. Washing 30 colonies ($\cong 10^5$ cells) with PBS free of calcium^{2+} (Ca^{2+}) and magnesium^{2+} (Mg^{2+}) (Gibco/BRL).

Table 1
Definitions to be used when performing relative quantification with LightCycler®

Term	Definition
Crossing point (Cp)	Crossing point is that where PCR amplification begins to become clearly positive above the background phase. Therefore, Cp is considered to be the most reliably proportional point to the initial concentration. Cp differs for each sample because different amounts of starting material need different numbers of cycles to reach this detection limit
Housekeeping gene	A gene that is not only expressed constitutively but also at the same level in all samples to be analyzed. It is used as a reference for mRNA quantification
Calibrator	A positive sample for all genes analyzed to which all other samples are compared. It is used: – To normalize sample values within a run and between runs, and it must keep a constant ratio of the target gene expression to the reference gene expression – To correct PCR efficiency
Standard curves	A series of calibrator dilutions which are used to generate standard curves for determining the efficiency of amplification in all the genes tested
Negative control	A non-template control. It is necessary to be included for every lot of reactions to check for contamination and reaction artifacts

2.2 RNA Extraction	1. Zymo extraction columns containing lysis buffer and washing buffer (Zymo Research).
	2. RNase-free water for resuspension and quantification (Gibco/BRL).
	3. RNase Inhibitor (Clontech, BD, Epicentre).
2.3 Retrotranscription	1. Eco-RT MMLV Retrotranscription Enzyme (Ecogen), Oligo-dT, dNTPs, Buffer 5× (Clontech, BD), RNase Inhibitor (Clontech, BD, Epicentre).
2.4 Primers	1. Stock solutions are provided in 100 µM (Sigma) concentration.
	2. Dilute to a working concentration of 10 µM with high purity water (Gibco/BRL). Prepare small quantities to avoid several freeze-thaw cycles that can disturb the quality of the primers.
2.5 Real-Time Kit	1. LightCycler FastStart DNA MasterPLUS SYBR Green I® (Roche) containing:
	(a) Vial 1a (white cap) containing the Taq DNA polymerase.
	(b) Vial 1b (green cap) reaction mix containing SYBR Green I®.
	(c) Vial 2 containing H_2O of PCR grade.
	(d) To prepare the Master Mix, pipette 14 µl from vial 1a into vial 1b. Each vial 1a contains enough enzyme for three vials of reaction mix. Mix gently by pipetting up and down.

Do not vortex. Relabel vial 1b (green cap) to Master Mix. Always keep the Master Mix away from light. The Master Mix can be kept at $-20\ ^{\circ}$C in the dark for 1 week.

2.6 Agarose Electrophoresis

1. TAE $10\times$ (Sigma), TAE $1\times$. Prepared with double distilled water.

2. Agarose 2 % (to visualize small amplicons), prepared in TAE $1\times$.

3. Loading Buffer Blue Juice ($10\times$) (Invitrogen, CA).

4. Ladder of 100 bps (Invitrogen, CA).

3 Methods

3.1 Material Preparation

3.1.1 hESC Cultures

hESC maintenance usually lasts over long periods, ranging from months to years of cultures. To assess their undifferentiation stage, samples must be obtained periodically. However, the minimum representative number of cells is a critical step. Common characterization processes in other cell cultures usually involve million-order figures. However, this is not a practical process to be applied routinely to hESC cultures, where conditions must be adjusted to take the minimum representative number of cells. In our laboratory practice, we have standardized 10^5 cells as an adjusted number to work with while performing RT-PCR for the established gene analysis. Monitoring is proposed every four to five passages, which could correspond to 1 month of passaging.

The determinant factors to be taken into account when routinely performing this technique involve a proper design of high-quality primers, setting up an appropriate calibrator, and a suitable housekeeping gene.

3.1.2 Preparation of RNA

This protocol is performed assuming that the Mini RNA Isolation Kit (Zymo Research) is based on a single-step RNA extraction/binding buffer combined with fast-spin column technology. This kit allows the efficient isolation of total RNA from 1×10^1 to 1×10^5 cells. However, other similar procedures have been demonstrated to work well (*see* **Note 1**):

1. Collect cells at $400 \times g$ for 5 min at room temperature.

2. Wash cells with PBS free from Ca^{2+} and Mg^{2+}.

3. Allow the supernatant to dry as much as possible. At this stage, cells can be frozen at $-80\ ^{\circ}$C, or the protocol should be followed.

4. Add 200 μl RNA extraction buffer to the cell pellet. Resuspend the cells gently by vortexing.

5. Incubate the lysate on ice for 20 min with brief vortexing every 10 min.

6. Add one volume (200 μl) 95–100 % ethanol to the lysate. Mix briefly and then incubate on ice for 10 min.

7. Transfer the mixture to a Zymo-Spin™ column in a supplied collection tube.

8. Spin in a microcentrifuge at $\geq 9,300 \times g$ for 1 min and discard the flow-through.

9. Add 200 μl RNA wash buffer to the column. Centrifuge at $\geq 9,300 \times g$ for 1 min and discard the flow-through.

10. Repeat step 9.

11. Transfer the column to a new RNase-free 1.5 ml microcentrifuge tube.

12. Add 10 μl RNase-free water directly to the column membrane. After 2 min, centrifuge at $\geq 9,300 \times g$ for 1 min to elute the RNA.

13. Quantify and assess the RNA quality in a Nanodrop® spectrophotometer (*see* **Note 2**).

14. The eluted RNA can be used immediately or stored at −80 °C for future use after adding 0.5–1 μl RNase Inhibitor (*see* **Notes 3** and **4**).

3.1.3 Preparation of cDNA

1. Prepare a mix containing:

Component	Volume
Total RNA	0.5–1 μg
Oligo(dT)$_{18}$	1 μl
Nuclease-free water	Up to 12 μl
Final volume	12 μl

2. Incubate the mix for 5 min at 70 °C, and then chill on ice.

3. Add the following:

Component	Volume
RNase Inhibitor	0.5 μl
dNTP mix 100 mM	1 μl
5× Reaction buffer	4 μl
Water	Up to 19.5 μl
Final volume	19.5 μl

*If more than one reaction is to be done, prepare a Master Mix by calculating volumes for $n + 1$, considering n the number of reactions.

4. Mix by pipetting.

5. Add in ice 0.5 μl of ECO-RT at 200 U/μl.

6. Incubate at 42 °C for 60 min.

7. Stop the reaction by heating at 70 °C for 10 min. Chill on ice.

8. Dilute the cDNA up to 100 μl, assess both quantity and quality in the Nanodrop®, and keep at −20 or −70 °C (*see* **Notes 2, 4, and 5**).

3.1.4   Primer Design

Real-time PCR results rely on the quality and the accuracy of the primers used; thus, very strict parameters for their design and subsequent validation are required. Although some primers used for end-point PCR primers are also useful for RT-PCR, specific primer sets must be designed in most cases for these conditions. The concentrations of PCR reactions, such as Taq DNA polymerase, $MgCl_2$, other salts, and dNTPs, remain constant within the same chemistry (SYBR Green), thus making primers the only point of flexibility in performing assays.

Primer sets must reach a single peak in the melting curve as it is indicative of a single PCR product. Multiple peaks in this plot indicate that nonspecific products or primer dimers have formed. The formation of a single product can also be confirmed by running the PCR products on 2 % agarose following the PCR run. The following steps must be taken into account for the proper design of primers:

1. Retrieve the sequence information from the appropriate source (e.g., GeneBank or Ensembl).

2. Determine the locations of exon boundaries by aligning the mRNA sequence with its gene (i.e., GenAtlas). Some genes do not have introns, so this step may not be applied.

3. Copy the sequence into the design software (Genefisher, Primer3) for 130–300 bps expected amplicons.

4. Perform a BLAST (or equivalent) search of both the primers of the set together to verify that they will fully anneal to the correct sequence, and only to that sequence.

5. Validate the primer set according to the remaining steps of this protocol.

3.1.5   Selection of a Calibrator

The calibrator is a positive control sample with a constant ratio of the target gene to the reference gene. It plays a double role in real-time PCR experiments. Firstly, it can be used to check PCR efficiency with primers. Secondly, it is usually employed as an internal control between PCR runs, thus allowing long-term studies when several LightCycler® runs must be compared over time.

In principle, any sample can be defined as the calibrator. However, it is highly recommended to choose a sample easy to obtain, since it needs to be included in every run. The calibrators suggested for hESC experiments include:

1. For undifferentiation genes: A hESC line.

2. For differentiation genes: Commercially available somatic tissues.

3. For housekeeping genes: Both the abovementioned must be positive for reference genes.

3.1.6 Selection of Reference Genes

Housekeeping genes are a large group of genes that code for proteins whose activities are essential for the maintenance of the cell function. The detection of housekeeping gene mRNA is routinely used to control several variables that may affect RT-PCR. These endogenous controls are constitutively expressed in each experimental sample and, therefore, serve as perfect candidates for the normalization of the final result.

Commonly used housekeeping genes include *GAPDH*, albumin, actins, tubulins, cyclophilin, and ribosomal genes (10–13). However, the expression level of housekeeping genes may vary depending on the cell type analyzed the extent of cell proliferation, differentiation, and the various experimental conditions. Specifically *GAPDH* and *ACTB*, which are generally established as housekeeping markers, have been demonstrated to display variations that are unacceptable for reference genes as they change at different developmental stages (14–16). Thus, ribosomal genes, like 18 s or Rpl19, are better endogenous reference controls for RT-PCR experiments.

3.1.7 Selection of Target Genes

Consensus genes for the stemness monitoring of long-term hESC cultures are specified in Table 2, along with the designed primers and the amplified fragments.

3.2 Experimental Setup

3.2.1 Establishing a Protocol

This procedure is available providing that a LightCycler 2.0® (Roche), or similar equipments, is used, as well as the elements included in the LightCycler FastStart DNA MasterPLUS SYBR Green I® (Roche).

LightCycler Protocol

Program the LightCycler Instrument before preparing the reaction mixes as indicated in Table 3.

Preparation of the PCR Mix

1. Place the required number of LightCycler® Capillaries in pre-cooled centrifuge adapters or in a LightCycler® Centrifuge Bucket (*see* **Notes 6–10**).

Table 2
Proposed primers for differentiation and undifferentiation genes

Gene	Characteristic	Primers name	Primers sequence	Fragment pbs
OCT3/4	Undifferentiation	OC2FR OC2RR	CGAAAGAGAAAGCGAACCAG GCCGGTTACAGAACCACACT	157
REX1	Undifferentiation	REX-51 REX-31	GGCGGAAATAGAACCTGTCA CTTCCAGGATGGGTTGAGAA	152
NANOG	Undifferentiation	NAN-FR6 NAN-RR6	CCGTTTTTGGCTCTGTTTTG TCATCGAAACACTCGGTGAA	187
KER	Ectoderm	KER-FR KER-RR	CAGATGCTGTGTCCCTGTGT TTCAGATCCAGAAGGGGATG	130
CMP	Mesoderm	CMP-FR CMP-RR	CCGGAGCCAGGACTACATTA GGTCTTGAAGTCAGCCGTGT	160
AFP	Endoderm	AFP-FR AFP-RR	ACACAAAAAGCCCACTCCAG GGTGCATACAGGAAGGGATG	147
AMY	Endoderm	AMY-FR AMY-RR	CATCTGTTTGAATGGCGATG TTCCCACCAAGGTCTGAAAG	138
RPL19	Housekeeping	19-FR3 19-RR3	CGAATGCCAGAGAAGGTCAC CCATGAGAATCCGCTTGTTT	157

2. In a 1.5 ml reaction tube on ice, prepare the PCR Mix for one or $n + 1$ (considering $n =$ number of reactions) as follows:

Component	Volume (μl)
H_2O PCR grade (vial 2)	12
Primer forward 10 μM	1
Primer reverse 10 μM	1
Master Mix, 5× conc (vial 1)	4
Final volume	18

3. Mix gently by pipetting up and down. Do not vortex.

   (a) Pipette 18 µl PCR Mix into each LightCycler Capillary.

   (b) Add 2 µl cDNA template (*see* **Note 11**).

   (c) Seal each capillary with a stopper.

4. Place the adapters containing the capillaries into a standard benchtop microcentrifuge and centrifuge at $800 \times g$ for 5 s.

5. Transfer the capillaries into the sample carousel of the Light-Cycler® Instrument and follow the "LightCycler® Protocol."

Table 3
PCR program parameters for a regular Lightcycer PCR run

Analysis mode	Cycles	Segment	Target temperature	Hold time	Acquisition mode
Denaturation					
None	1		95 °C	7 min	None
Amplification					
Quantification	35–45	Denaturation	95 °C	10 s	None
		Annealing	Primer dependent[a]	0–10 s[b]	None
		Extension	72 °C	=amplicon (bp)/25 s	Single
Melting curve					
Melting curves	1	Denaturation	95 °C	0 s	None
		Annealing	65 °C	15 s	None
		Melting	95 °C	0 s	Continuous
			Slope = 0.1 °C/s[c]		
Cooling					
None	1		40 °C	30 s	None

[a]For initial experiments, set the target temperature 5 °C below the calculated primer Tm. Calculate the primer Tm according to the following formula: Tm 2 °C (A + T) + 4 °C (G + C)
[b]For typical primers, choose an incubation time of 0–10 s for the annealing step. To increase the specificity of primer binding, use an incubation time of <5 s
[c]In all other cases, the slope will be 20 °C/s

Standard Curves:
Correction of Efficiency

The accuracy of quantification depends solely on the differences in the amplification of the target and reference genes. To determine efficiency, we must construct a relative standard curve (log concentration of standard dilutions vs. cycle number). The slope of that curve can be directly converted into efficiency by the formula $E = 10^{-1/\text{slope}}$.

A slope of -3.32 indicates the maximum possible reaction efficiency (2.0), which means that the amount of PCR product doubles during each cycle. However, amplification reactions operate at less than maximum efficiency because experimental conditions are not fully optimized and samples can contain PCR inhibitors. Thus, if efficiency is assumed to be 2 when PCR efficiency is less, an error is introduced that proportionally increases with the cycle number. For example, if there is a difference in the amplification efficiency of 0.05 between the target and reference genes, the calculated relative target value can be more than twofold (113 %) off if it is calculated after 30 cycles. Therefore, PCR efficiency correction should always be considered.

To assess the PCR efficiency correction, standard curves will not only be performed with the calibrator for all the target gene primers analyzed but also with the reference primers (Fig. 1).
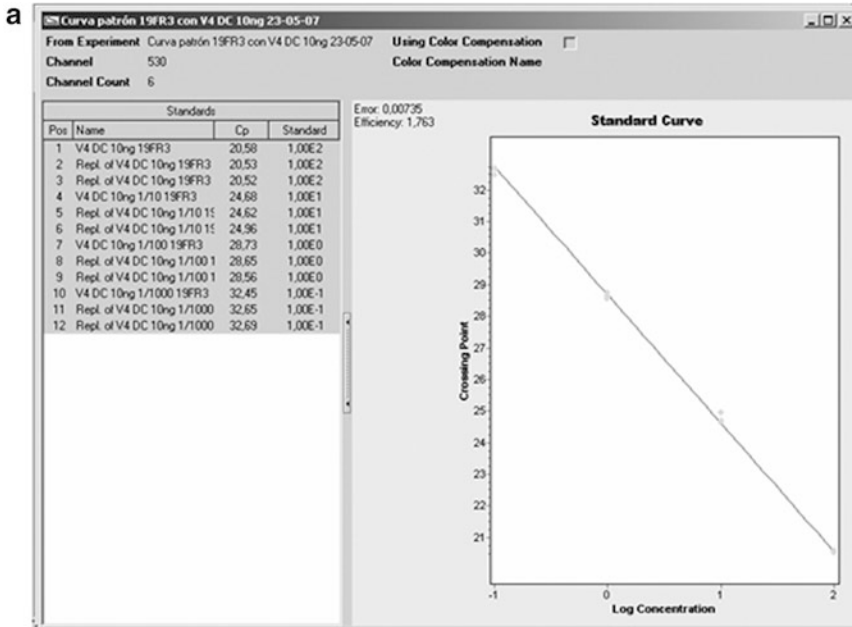
Fig. 1 Calibration curve with all the gene markers proposed to monitor the hESC lines. (**a**) Example of a calibration curve with the housekeeping gene RPL19 using serial dilutions of differentiated VAL-4 (V4DC) as a template [5]. (**b**) PCR efficiencies for all the target and reference genes using the calibrator as a template for both the undifferentiation (VAL-4 P42) and differentiation markers (VAL-4 DC)

For a statistically valid standard curve, the standard dilutions should span at least 3–5 orders of magnitude, and all dilutions should be within the same concentration range as the unknown ones to be analyzed. In addition, use a minimum of four to five serial dilutions (e.g., 1:10 dilutions) for each curve, and prepare at least three replicates of each. Replicates are especially important for lower concentrations since the Cp distortion increases at low concentrations.
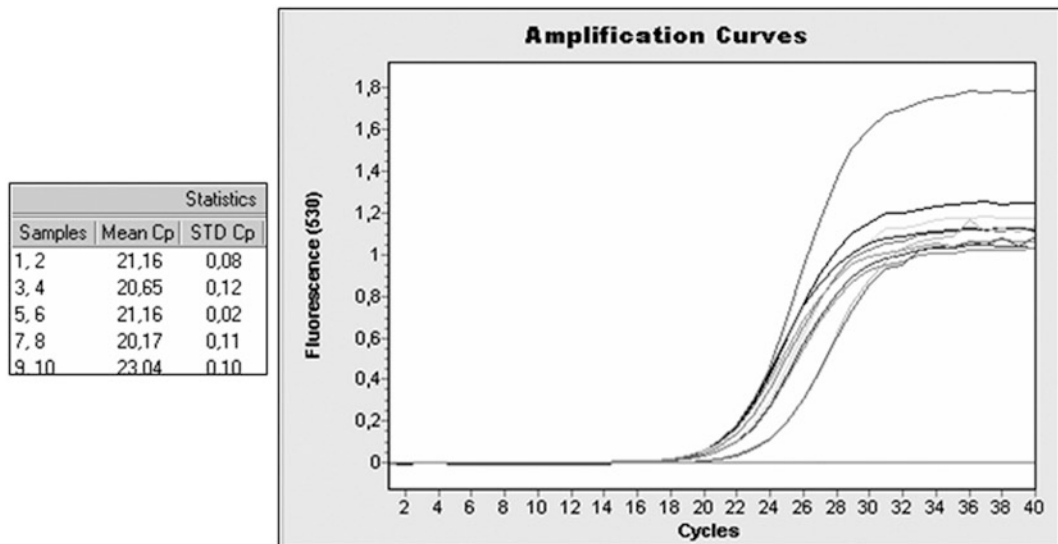
Fig. 2 Absolute quantification for the Oct4 experiment. Samples (1–8) and the calibrator VAL-4 P42 (9, 10) were analyzed per duplicate by the Crossing point (Cp) detection. The negative control was also included as sample 11 and corresponds to the *flat line* in the graph. All positive samples have a logarithmic exponential phase and a final plateau phase

| Experimental Design | Each experimental lot will include: |

Experimental Design

Each experimental lot will include:

1. Per gene analyzed:
   (a) Sample/samples to be analyzed (×2)
   (b) Calibrator (×2)
   (c) Negative control (water ×1)
2. Per passage analyzed:
   (a) Undifferentiation gene markers: *OCT-4*, *REX-1*, *NANOG*.
   (b) Differentiation gene markers: *KER*, *CMP*, *AMY*, *AFP*.
   (c) Housekeeping gene marker: *RPL19*.

Data Analysis

Absolute Quantification

The LightCycler® program firstly allows to quantify by measuring the Cp of each sample (*see* **Note 12**). This value will be that which is to be normalized with both the reference and calibrator for the relative quantification study (Fig. 2).

Specificity Assessment

1. Melting Curves
   Melting curves allow to check for amplicon specificity. Each fragment amplified has a unique and specific melting temperature (Fig. 3).

2. Agarose Electrophoresis
   Apart from the melting curve, samples can be separated on an agarose gel (2 %). If this is done immediately after the PCR run,
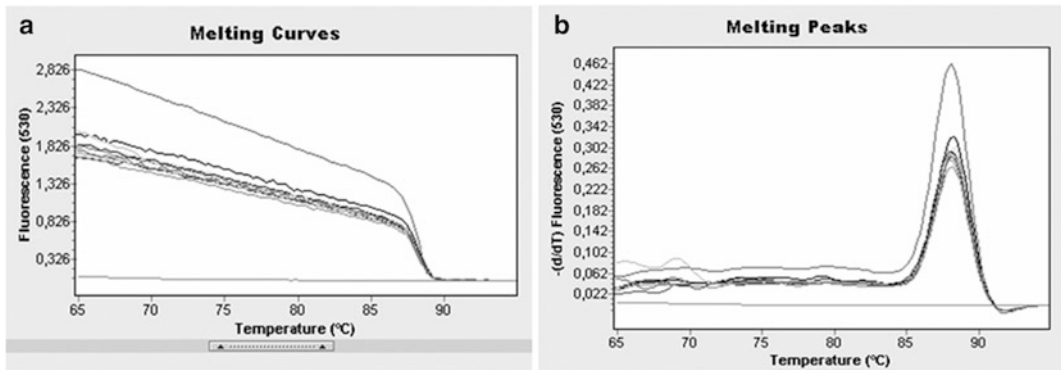
Fig. 3 (**a**) Melting curves of Oct4 primers with VAL-4 samples. (**b**) Positive samples have a single peak of 88 °C. The negative control has no melting peak

no ethidium bromide is necessary as SYBR Green can also be visualized with UVA irradiation.

Relative Quantification

1. LightCycler® Software
   The results are expressed as the target/reference ratio of each sample normalized by the target/reference ratio of each sample normalized by the target/reference ratio of the calibrator when analyzed for Relative Quantification Monocolor using the LightCycler® software (*see* **Notes 12** and **13**).

   If replicates are included in the run, the software calculates and displays the median of the Cps from those replicates and the resulting Cp median values [Cp (median target) minus Cp (median of reference)]. The last two columns of the results screen show the ratio concentration [(target concentration) divided by (reference concentration)] and the final result, the normalized ratio [(ratio conc. of sample divided by ratio conc. of calibrator)], which is the valid one (Fig. 4).

3.3 Interpretation of the Results

Following the assay, the resulting raw data are analyzed using second-party software, usually Microsoft Excel or equivalent. The data analyses are dependent on the type of assay performed and are outlined in detail as part of each protocol (*see* **Note 14**). RT-PCR allows the quantification of the gene expression over time when compared to a proper reference gene and calibrator. Undifferentiation markers must be present and in a similar ratio to that of the reference gene and must always be expressed constitutively, irrespectively of the culture conditions or cell cultures aging (Fig. 5a). Early differentiation markers must be absent in hESC cultures or at very low expression levels, thus showing that stemness still remains in long-term hESC cultures (Fig. 5b).
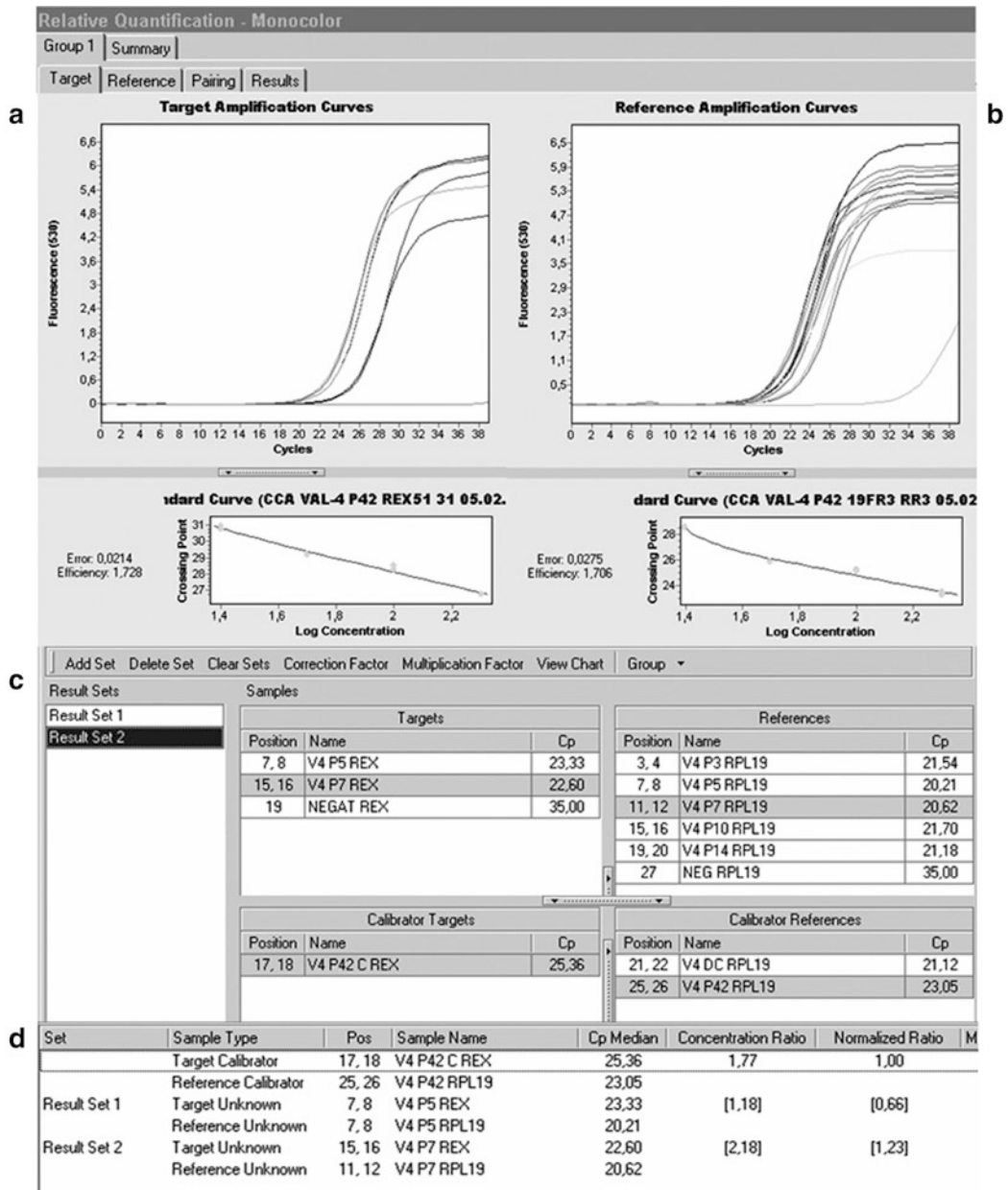
Fig. 4 Relative Quantification Monocolor, a four-step procedure. (**a**) The first step consists in introducing data related to target Cp values and in importing the external standard curve performed with the calibrator. (**b**) The second step consists in introducing the corresponding reference data of the same samples in step (**a**) and also importing the external standard curve for the reference gene with the same calibrator. (**c**) The pair results of the median Cp of the target and reference samples and their corresponding calibrator data. (**d**) Normalized ratio results considering the PCR efficiency
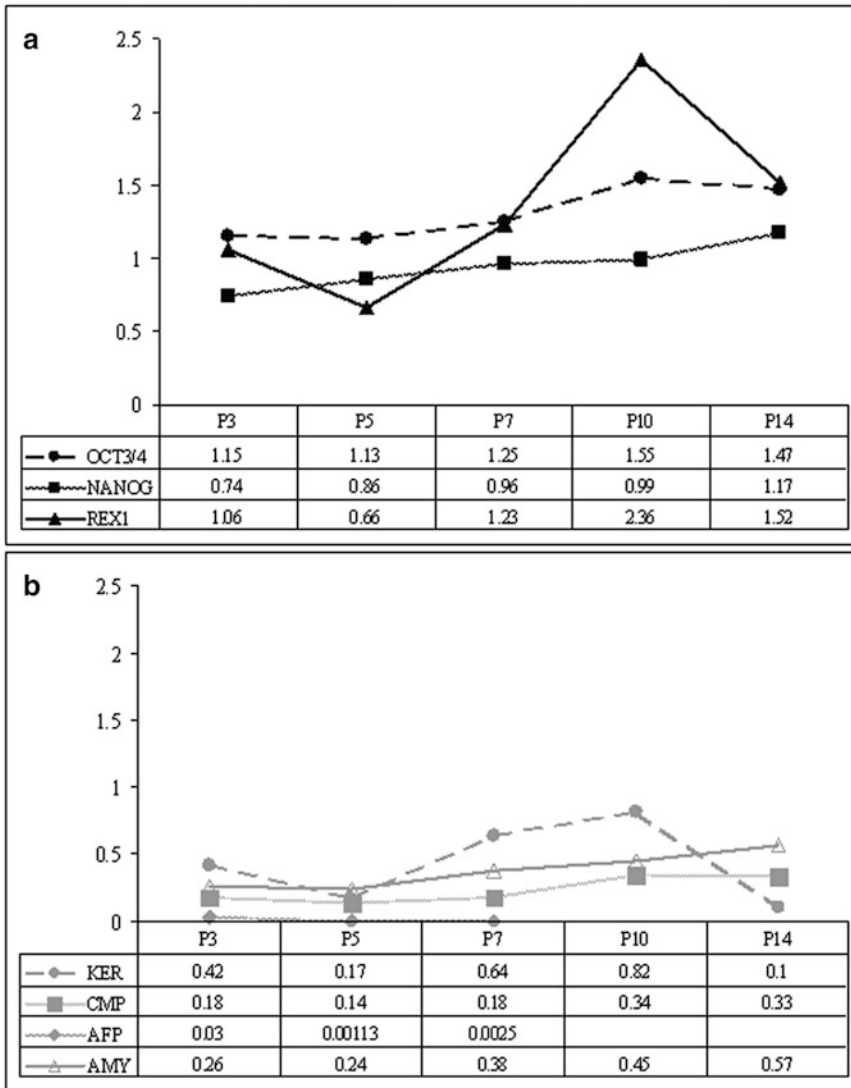
Fig. 5 Gene expression monitoring is the result of the relative quantification of selected undifferentiation (**a**) and differentiation (**b**) markers. The VAL-4 hESC line was analyzed in passages 3, 5, 7, 10, and 14 to monitor its undifferentiation and differentiation stage for more than a 2-month period. The data values indicate that the hESC line maintains its properties of stemness throughout the passages

4   Notes

1. Unless stated otherwise, always use RNase-free water (i.e., Gibco/BRL); treat the gel tank and cassette, the bench, and other surfaces with RNase ZAP (Sigma), and use gloves and lab coat, and aerosol-resistant filter tips if possible.

2. Use high-quality RNA and cDNA. Whenever possible, use a ratio of absorbance at 260/280 of ~1.8 for DNA and a ratio of

~2.0 for RNA. The 260/230 ratio may commonly be in the range of 1.8–2.2.

3. Aliquot RNA in the case of the calibrator and add RNase Inhibitor to keep RNA at −80 °C for long-term storage.

4. Follow the same RNA extraction procedure and cDNA synthesis for all samples analyzed, and in the same lot of experiments, if possible.

5. Perform a two-step reverse transcription PCR to obtain enough cDNA for all the experiments.

6. Aliquot primers to prevent contamination and freeze/thaw cycles that inhibit their efficiency.

7. Keep the reaction mix, once prepared, for a maximum of 1 week at −20 °C.

8. Do not touch the surface of the capillaries and always wear gloves when handling them.

9. Perform each step of the experiment in different areas to avoid cross-contamination. Use a hood to prepare real-time reactions. Run reactions on a separate bench, and perform runs when electrophoresed in agarose separately from where real-time reactions are being prepared.

10. Sterilize the pipettes, the filter tips, and the bucket by UVA irradiation in a hood previous to use.

11. Fill real-time reactions starting from the negative control (water) followed by the most diluted template, and finish with the most concentrated sample to avoid cross-contamination.

12. Since the result is expressed as a ratio, relative quantification assays are more or less independent of the initial amount of template. However, each sample must contain enough sample material to be detectable and to generate Cp values during the run.

13. The target and the reference genes are theoretically expressed at a constant ratio in the calibrator sample. However, this ratio may slightly fluctuate in different calibrator batches. For data consistency, it is very important to define a batch-specific correction factor to compensate for such batch-to-batch differences.

14. An enormous amount of data is generated when monitoring several passages, hESC lines, and genes. Therefore, an accurate evaluation by statistical analysis is necessary. Depending on the individual assay or system under investigation, various methods and/or programs may be used, including the classical standard analysis of variance (e.g., the Student's t-test), Poisson's error law, or other applied statistical systems available in the literature or on the Internet which are suitable for each requirement.

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Study of Gap Junctions in Human Embryonic Stem Cells

Alice Pébay and Raymond C.B. Wong

Abstract

Gap junctional intercellular communication (GJIC) has been described in different cell types including stem cells and has been involved in different biological events. GJIC is required for mouse embryonic stem cell maintenance and proliferation and various studies suggest that functional GJIC is a common characteristic of human embryonic stem cells (hESC) maintained in different culture conditions. This chapter introduces methods to study gap junctions in hESC, from expression of gap junction proteins to functional study of GJIC in hESC proliferation, apoptosis, colony growth, and pluripotency.

Keywords: Connexin, Gap junction, Gap junctional intercellular communication, Human embryonic stem cells

1 Introduction

Gap junctions are intercellular channels consisting of two connexons localized in the membrane of adjacent cells. Each connexon consists of six membrane proteins, termed connexins (Cx) (1, 2). Numerous molecules can diffuse through gap junction channels, including small ions, second messengers, amino acids, metabolites, short interfering RNA, and peptides involved in cross-presentation of major histocompatibility complex class I molecules (3–6). Such intercellular coupling is termed gap junctional intercellular communication (GJIC). GJIC is involved in various cellular mechanisms, including control of cell migration, proliferation, differentiation, metabolism, apoptosis, and carcinogenesis (4, 7–11). In human embryonic stem cells (hESC), various connexins have been found to be expressed: Cx25, Cx26, Cx30, Cx30.2, Cx30.3, Cx31, Cx31.1, Cx31.9, Cx32, Cx36, Cx37, Cx40, Cx43, Cx45, Cx46, Cx47, Cx59, and Cx62 (12–14, 15). Among these connexins, Cx43 and Cx45 are highly enriched in undifferentiated hESC compared to its differentiated counterpart in many microarray studies, and the two connexins were identified as "undifferentiated hESC markers" (16). Moreover, Cx43 was identified as a downstream

target of key pluripotent transcription factors Oct4, Sox2, and Nanog (17). Undifferentiated hESC can communicate through functional GJIC, as determined by dye coupling (12–14) and ionic coupling (15). Finally, functional GJIC is a common characteristic of undifferentiated hESC maintained in different culture conditions, suggesting an understudied role of gap junctions in mediating hESC maintenance (13, 14). Studies of intercellular communication through gap junctions can potentially lead to novel methods to improve clonal survival and maintenance of hESC, which is fundamental to realize the therapeutic potential of these cells.

2 Materials

2.1 General (See Note 1)

1. hESC were cultivated in different formats depending on the experiments to perform. In all cases, we culture hESC with a feeder layer of mouse embryonic fibroblasts (MEF) supplemented with 20 % fetal calf serum or 20 % Knockout serum replacement (Invitrogen, #10828-028) plus 4 ng/ml bFGF (R&D, #233-FB-025/CF).

2. For reverse transcriptase-polymerase chain reaction (RT-PCR), western blot, cell proliferation assay, cell pluripotency assay, and colony growth assay, we culture hESC in center-well organ culture dishes, 60×15 mm style (35 mm culture dishes, Falcon, #353037).

3. Alternatively for western blot, we culture hESC with MEF in 6-well plates (Falcon, #353046).

4. For immunocytochemistry and SL/DT, we culture hESC in Lab-Tek Chamber slide w/cover, permanox slide sterile (8-well chamber slides, Lab-Tek, #177445).

5. TrypLE Express (Invitrogen, #12604).

6. Phosphate-buffered saline (PBS) $10\times$: 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4.

7. Dispase (Invitrogen, #17105-041).

8. Ethanol and Methanol (Merck).

9. Tris base (Merck).

2.2 RT-PCR

1. RNase-free microtubes and pipette tips.

2. Dynabeads® Oligo $(dT)_{25}$ (Invitrogen, Dynal, #610.02, 610.05, 610.50) and Magnetic Particle Concentrator (Invitrogen, Dynal, #120.20D).

3. Lysis/binding buffer: 20 mM Tris–HCl (pH 7.5), 1 M LiCl, 2 mM EDTA.

4. Washing buffer A: 10 mM Tris–HCl (pH 7.5), 0.15 M LiCl, 1 mM EDTA, 0.1 % LiDS.

5. Washing buffer B: 10 mM Tris–HCl (pH 7.5), 0.15 M LiCl, 1 mM EDTA.

6. Superscript II Rnase H⁻ Reverse Transcriptase (Invitrogen, #18064-014).

7. TAQ DNA polymerase (Biotech International, #TAQ-1).

8. dNTP mix.

9. Sense and antisense primers for Cx43 and Cx45: Cx43, sense 5′-ATGAGCAGTCTGCCTTTCGT-3′, antisense 5′-TCTGC TTCAAGTGCATGTCC-3′; Cx45, sense 5′-GGAAGATGGG CTCATGAAAA-3′, antisense 5′-GCAAAGGCCTGTAAC ACCAT-3′.

10. Agarose molecular biology grade (Scientifix, #9010B).

11. TAE buffer: 20 mM Tris–Cl, pH 7.8, 10 mM sodium acetate, and 0.5 mM EDTA.

12. Ethidium bromide (Sigma, #E1510).

13. MinElute Gel Extraction Kit (Qiagen, #28604).

2.3 Western Blot Analysis

1. Mini-protean 3 system (Biorad, #165-3301, 165-3302, 170-3930, 170-3935).

2. 30 % Acrylamide/Bisacrylamide solution 29:1 3.3 % C (w/v) (Biorad, #161-056): Toxic product that should be used with care under a fume hood.

3. Resolving gel buffer: 1.5 M Tris–HCl, pH 8.8.

4. Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8.

5. Ammonium persulfate (APS, Biorad, #161-0700): Prepare fresh solution of APS 10 % in water.

6. TEMED (Biorad, #161-0800).

7. Laemmli sample buffer (Biorad, #161-0737) or reducing sample buffer: 0.1 M Tris–HCl (pH 6.8), 41.6 % (v/v) glycerol, 3.3 % (w/v) SDS, 0.02 % (w/v) bromophenol blue. Add 0.46 mM β-mercaptoethanol (Sigma, #M7154), 1 mM Sodium orthovanadate (Sigma, #S6508), and 1 mM Phenyl-methylsulfonyl fluoride (PMSF, Sigma, #P7626) to the sample buffer prior to cell lysis.

8. Kaleidoscope prestained ladder (Biorad, #161-0324).

9. Running buffer 1×: 25 mM Tris base, 192 mM glycine, 0.1 % SDS (w/v), pH 8.3. Mix 3.03 g Tris base, 14.4 g glycine, and 1 g SDS, bring final volume to 1 l with distilled water, and check pH.

10. Transfer buffer 1×: 25 mM Tris base, 192 mM glycine, 20 % methanol, 0.1 % SDS (w/v), pH 8.3. Mix 3.03 g Tris base, 14.4 g glycine, 200 ml methanol, and 1 g SDS, bring final volume to 1 l with distilled water, and check pH.

11. Hybond-P PVDF membrane (Amersham Pharmacia Biotech #RPN 303F) or PVDF membrane (Biorad #162-0177) cut to the size of the gel or precut (ready gel Blotting Sandwiches, Biorad, #162-0219).

12. Bovine serum albumin (BSA) fraction 5, min 96 % (Sigma, #A4503).

13. Tris-buffered saline with Tween 20 (TBST) pH 7.6: 20 mM Tris base, 137 mM sodium chloride, 3.8 mM HCl, 0.05 % Tween-20 (Biorad, #170-6531). Mix 2.42 g Tris base, 3.8 ml HCl 1 M, and 0.5 ml Tween-20, bring final volume to 1 l with distilled water, and check pH.

14. Rabbit anti-mouse Connexin 43 affinity-purified polyclonal antibody (Chemicon, #AB1728).

15. Negative control Rabbit Immunoglobulin Fraction, Solid-Phase Absorbed (Dako, #X0936).

16. Mouse monoclonal anti-β-tubulin 1 (Sigma #T7816).

17. Polyclonal Goat Anti-Rabbit Immunoglobulins/Horseradish peroxidase (HRP) (Dako, #P0448).

18. ECL Plus (Amersham Pharmacia Biotech #RPN2132, RPN2133) or Pierce ECL western blot substrate (chemiluminescent detection reagent, Pierce #32209).

19. Hyperfilm (Amersham Biosciences) or Gel-Doc system (Biorad).

20. Western Blot Stripping Buffer (Pierce, #21059).

21. Ponceau S (Sigma, #P-3504).

2.4 Immunocyto-chemistry

1. Glass cover slips 22 × 60 mm.

2. Wax pen/liquid blocker.

3. Rabbit anti-mouse Connexin 43 affinity-purified polyclonal antibody (Chemicon, #AB1728).

4. Rabbit anti-Connexin 45 polyclonal antibody (Chemicon, #AB1745).

5. 6-Diamidino-2-phenylindole (DAPI, Sigma, #D9542) or Bisbenzimide H 33342 (Hoechst-33342, Sigma, #382065): Prepare a fresh solution at 1 μg/ml in water.

6. Mouse GCTM-2 antibody (gift from Prof M. Pera, University of Melbourne).

7. Mouse anti-Oct-3/4 C-10 antibody (Santa Cruz Biotechnology, Inc. #sc-5279).

8. Mouse TG-30 antibody, recognizing CD9 (gift from Prof M. Pera, University of Melbourne).

9. TRA-1-60 antibody (gift from Prof P. Andrews, University of Sheffield).

10. Negative control Rabbit Immunoglobulin Fraction, Solid-Phase Absorbed (Dako, #X0936).

11. Negative control Mouse IgM antibody (Dako, #X0942).

12. FITC-conjugated Swine Anti-Rabbit Immunoglobulins (Dako, #F0205) or Alexa Fluor® 488 goat anti-rabbit IgG (H + L) (Invitrogen, #A11008).

13. FITC-conjugated Rabbit Anti-Mouse Immunoglobulins (Dako, #F0261) or Alexa Fluor® 568 goat anti-mouse IgG (H + L) (Invitrogen, #A11004).

14. Vectashield (Vector Laboratories, # H-1000).

15. Nail varnish.

2.5 Scrape Loading/ Dye Transfer Assay

1. Ca^{2+}- and Mg^{2+}-PBS buffer (buffer 1): 140 mM NaCl, 5.5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM Hepes, pH 7.35.

2. Lucifer Yellow CH dipotassium salt (Sigma, #L0144) to freshly dilute in buffer 1 (1 mg/ml).

3. Dextran, tetramethylrhodamine (Rhodamine dextran, Molecular Probes, #D1868) to freshly dilute in buffer 1 (1 mg/ml).

4. Surgical blades (Swann-Morton, #0201).

2.6 Chemical Closure of Gap Junctions

1. Phorbol 12-Myristate 13-acetate (PMA, Sigma, #P8139).

2. U0126 (Promega, #V112A).

3. α-Glycyrrhetinic acid (Sigma, #G8503).

4. Recombinant human BMP-4 (R&D, #314-BP-010).

2.7 Cell Apoptosis Assay

1. In situ Cell Death Detection Kit, Fluorescein (Roche, #11684795910), containing TUNEL reaction mixture.

2. 2 % (w/v) Paraformaldehyde (PFA) in PBS: Stock can be aliquoted and stored at −20 °C. Once thawed, aliquots should be used on the day and discarded.

3. 0.1 % Triton X-100 in PBS.

2.8 Cell Proliferation Assay

1. In situ Cell Proliferation Kit, FLUOS (Roche, #1810740), containing 10 mM BrdU, mouse anti-BrdU antibody conjugated to fluorescein.

2. 4 M HCl solution.

3. 70 % Ethanol.

4. Propidium iodide (Sigma, #70335).

2.9 Cell Pluripotency Assay	1. TRA-1-60 antibody (Santa Cruz, #sc-21705).
	2. TRA-1-81 antibody (Santa Cruz, #sc-21706).
	3. Goat serum (Invitrogen, #16210-046).
	4. Goat anti-Mouse IgM antibody conjugated to Alexa fluor 488 (Invitrogen, #A21042).
	5. Negative control Mouse IgM antibody (Dako, #X0942).

2.10 Image and Data Analysis	1. Inverted fluorescence microscope.
	2. Western blot analysis: Scion image software (NIH) or Gel-Doc system (Biorad).
	3. Statistical analysis: Graphpad Prism.
	4. Cell proliferation, apoptosis, pluripotency assay: Flow cytometer (Moflo, DIVA, or FC500).
	5. Colony growth assay: Analysis^B software (Olympus Software Imaging Solutions) or Adobe Photoshop.

3   Methods

3.1 Reverse Transcriptase-Polymerase Chain Reaction for Connexin mRNA Expression

1. Pre-warm dispase (10 mg/ml of culture medium) to 37 °C.

2. Harvest 10–12 day-8 hESC colonies by dispase treatment (10 min, 37 °C, *see* **Note 2**), and transfer cells to an Eppendorf tube.

3. Wash colonies four times with PBS. Centrifuge at $300 \times g$ for 2 min to spin down cells.

4. Isolate poly-A+ mRNA using Dynabeads Oligo $(dT)_{25}$ adapted from the supplier's instructions (*see* **Note 3**). Using the magnetic particle concentrator, all Dynabead-binded mRNA are captured to one end of the tube, and the mRNA-free supernatant is discarded.

5. Add 300 μl of the lysis/binding buffer to hESC extracts in an Eppendorf tube and homogenize with a pipette until complete lysis.

6. Transfer 20 μl of homogenized Dynabeads into an Eppendorf tube, and place onto the magnetic particle concentrator for 2 min. Remove supernatant, resuspend the beads in 200 μl of lysis/binding buffer, place onto the magnetic particle concentrator for 2 min, and remove supernatant.

7. Mix lysed hESC with Dynabeads, incubate for 10 min at room temperature, then place onto the magnetic particle concentrator for 2 min, and remove supernatant.

8. Mix 400 μl of washing buffer A with the beads, place onto the magnetic particle concentrator for 2 min, and remove supernatant.

Table 1
Reverse transcription buffer for cDNA synthesis

Reagents	RT$^+$ Volume (μl)	RT$^-$ Volume (μl)	Final concentration
5× First Strand Buffer (Invitrogen)	4	4	1×
Dithiothreitol (DTT)	2	2	0.01 M
dNTP mix[a]	1	1	0.5 mM
Superscript II reverse transcriptase	1		200 Units
MilliQ	12	13	

[a]dNTP mix contained dATP, dCTP, dGTP, dTTP

9. To enhance the purity of the mRNA preparation, repeat **step 8** first with 400 μl of washing buffer A and then twice with 200 μl of washing buffer B.

10. Add 200 μl of washing buffer B to the beads, mix thoroughly, and separate into two Eppendorfs, each containing 100 μl of washing buffer B and beads. Place onto the magnetic particle concentrator for 2 min, and remove supernatant. Label one tube as +RT and the other tube as −RT.

11. Perform RT using Superscript II Rnase H$^-$ Reverse Transcriptase, following the supplier's protocol. A negative control without the addition of reverse transcriptase (RT$^-$) must be performed for each RNA sample to check the absence of contaminating genomic DNA. A 20 μl reaction volume is prepared as follows (Table 1) and added to the +RT and −RT tubes.

12. Incubate for 1 h at 42 °C, then place on ice, mix solution, place onto the magnetic particle concentrator for 2 min, and remove supernatant. Resuspend in 20 μl distilled/RNase-free water.

13. Perform PCR experiments using Taq DNA polymerase. A negative control (−RT) must be performed for each cDNA sample. A water control (no cDNA) should also be included. For each reaction a total volume of 25 μl is prepared as shown in Table 2. For the cDNA samples, homogenize the preparation (cDNA and beads) before pipetting. It is recommended to perform PCR immediately after RT.

14. PCR reaction is performed with the following conditions using the specific primers for Cx43 and Cx45: initial denaturation at 94 °C for 5 min, 35 cycles of "denaturation at 94 °C for 30 s, annealing at 55 °C for 2 min, and extension at 74 °C for 2 min," ending with a final incubation at 74 °C for 7 min.

15. Amplicons are sized by electrophoresis on 2 % (w/v) agarose gel stained with 0.001 % (v/v) ethidium bromide in TAE buffer.

Table 2
PCR reaction mix

Reagents	cDNA Volume (μl)	−RT Volume (μl)	Water control Volume (μl)	Final concentration
10× Buffer	2.5	2.5	2.5	1×
dNTP mix	0.5	0.5	0.5	0.2 mM
Taq polymerase	0.25	0.25	0.25	0.25 Units
cDNA	3	0	0	
−RT	0	3	0	
Primer mix	2	2	2	2 μM
MgCl$_2$	1.5	1.5	1.5	1.5 μl
MilliQ	Make up to 25	Make up to 25	Make up to 25	

Table 3
SDS-polyacrylamide gel formulations

Percent of gel	MilliQ H$_2$O (ml)	30 % Acrylamide/Bis (ml)	Gel buffer (ml)	10 % w/v SDS (ml)
4 %	6.1	1.3	2.5[a]	0.1
10 %	4.1	3.3	2.5[a]	0.1

[a]Resolving gel buffer = 1.5 M Tris–HCl, pH 8.8
Stacking gel buffer = 0.5 M Tris–HCl, pH 6.8

16. Optional: Confirmation of the identity of amplicons: Excise DNA fragments of interest from the gels using a scalpel with the aid of a UV transilluminator; purify the amplified products using the QIAquick gel extraction kit following the supplier's instructions, and confirm the identity of the purified amplicons by DNA sequencing.

3.2 Western Blot Analysis of Connexin 43 Phosphorylation States: SDS-Polyacrylamide Gel Electrophoresis (See Note 4)

3.2.1 SDS-PAGE Gel Preparation

1. Western blot analysis is carried out using the Mini-protean 3 system, following the supplier's specifications.

2. Prepare a 10 % resolving gel and a 4 % stacking gel (Table 3). TEMED and freshly prepared 10 % (w/v) APS are to be added immediately prior to pouring the gel in order to catalyze polymerization.

   (a) Resolving gel: 50 μl 10 % (w/v) APS and 5 μl TEMED.

   (b) Stacking gel: 50 μl 10 % (w/v) APS and 10 μl TEMED.

3. Pour the 10 % resolving gel into a prepared gel cassette, leaving space for the stacking gel, overlay with water, and allow polymerization for ~40 min at room temperature.

4. Pour the 4 % stacking gel, add combs, and allow polymerization for ~40 min at room temperature.

3.2.2 Preparation of Cell Lysates

1. Harvest day-8 hESC colonies using dispase (10 min, 37 °C, *see* **Note 2**). Transfer cells to an Eppendorf tube.

2. Dilute 1:1 with commercially available Laemmli sample buffer or with a reducing sample buffer containing β-mercaptoethanol supplemented with 1 mM sodium orthovanadate and 1 mM PMSF.

3. Boil samples for 4 min and centrifuge for 10 min at 16,110 × *g*.

4. Samples should be kept at −80 °C for long-term storage. Avoid freeze-thawing samples, as this might impact on the dephosphorylation of the samples. Instead, aliquot samples.

3.2.3 Gel Electrophoresis and Transfer

1. Protein extracts (25 μl/well) are resolved on an SDS-polyacrylamide gel electrophoresis (PAGE) gel in running buffer at 200 V for ~35 min using a kaleidoscope prestained ladder (8 μl/well) to estimate the size of the resultant bands.

2. After separation, remove stacking gel with a surgical blade and transfer proteins in the resolving gel to a PVDF membrane. Do not touch the PVDF membranes with hands; use forceps.

3. Prior to use, the PVDF membrane must be activated with 100 % methanol for 30 s followed by a rinse in distilled water.

4. For optimum transfer, pre-equilibrate gels and the PVDF membrane in transfer buffer for at least 10 min prior to transfer.

5. Prepare a "gel sandwich" with pre-wet filter papers, the gel and the membrane in between, according to the manufacturer's instructions. Make sure that there is no air space (bubbles) between gel and membrane to ensure a good transfer.

6. Allow transfer for 1 h at 100 V in transfer buffer.

7. After transfer, remove the membrane and orientate it.

3.2.4 Immunoblotting and Protein Detection

1. All steps are performed on a rocking platform.

2. Block the PVDF membrane with 1 % BSA (*see* **Note 5**) in TBST either overnight at 4 °C or 1 h at room temperature.

3. Incubate the membranes with the primary rabbit polyclonal antibody against Cx43 (0.5 μg/ml in TBST) for 2 h or overnight. Negative control membranes should be incubated with the appropriate immunoglobulin-negative fraction at the same concentration.

4. Wash membranes three times in TBST (15 min each).

5. Incubate the membranes with the HRP-conjugated secondary antibody for 1 h at room temperature (0.15 μg/ml in TBST).

6. Wash membranes three times in TBST (15 min each).

7. Incubate the membranes with the chemiluminescent detection reagent for 5 min and expose to Hyperfilm. Hyperfilm exposure time must be optimized for the highest signal-to-noise ratio. Alternatively, chemiluminescence can be detected using the Gel-Doc system (Biorad). Cx43 appears as a triplet of band at approximately 43 kDa.

3.2.5 Membrane Stripping

1. Antibodies on the membrane can be stripped by incubation with the western blot stripping buffer (15–30 min at room temperature).

2. Following TBST washes, the membrane can be blocked and re-probe membranes with another antibody of interest, as described above.

3. Beta-tubulin antibody (1/10,000 in TBST for 1 h) followed by HRP-conjugated secondary antibody can be used as a lysate loading control, molecular weight 55 kDa (*see* **Note 6**).

3.3 Immunocyto-chemistry for the Expression of Cx43, Cx45, and Pluripotency Markers

1. Wash cells in PBS.

2. Fix cells in cold 100 % ethanol for 10 min at room temperature. Allow air-drying of the samples.

3. With a wax pen, delimit each individual well (to limit risks of "cross contamination" of reagents).

4. Wash cells three times in PBS.

5. Samples should be kept at −80 °C for long-term storage.

6. Block sample with 1 % serum for 1 h at room temperature (*see* **Note** 7).

7. Incubate cells with the following primary antibodies: Cx43 (20 μg/ml, 1/50–1/100 in PBS containing 0.1 % serum) and Cx45 (1/50–1/100 in PBS containing 0.1 % serum) for 30–60 min at room temperature. Negative control should be performed using sample incubated with the appropriate antibody isotype control.

8. Wash samples three times in PBS.

9. Incubate the samples with the appropriate secondary antibody conjugated with FITC (20 μg/ml, 1/40) or Alexa Fluor® 488 (6.67 μg/ml) for 30 min at room temperature.

10. Optional: To assess pluripotency, double staining can be performed using specific hESC markers such as the following antibodies: GCTM-2 (undiluted hybridoma supernatant), Oct-4 (4 μg/ml, 1/50 in PBS), TG-30/CD9 (undiluted hybridoma supernatant), and TRA-1-60 (undiluted) for 30 min at room temperature, followed by incubation with the appropriate secondary antibody conjugated with Alexa Fluor® 568 (6.67 μg/ml) for 30 min at room temperature.

11. Wash samples three times in PBS.

12. Counterstain nuclei with DAPI or Hoechst-33342 (1 µg/ml in water) for 5 min at room temperature.

13. After PBS washes, mount samples in Vectashield to enhance visualization of the immunostaining.

14. Cover the slide with a glass cover slip and seal with nail varnish.

15. Cx43 staining appears as a dotted staining at the membrane of cells, while we observed Cx45 staining to be at the membrane and the cytoplasm of hESC. Specificity is verified by the absence of immunostaining in the antibody isotype controls.

3.4 Scrape Loading/ Dye Transfer Assay (See Notes 8 and 9)

1. GJIC in hESC is determined using the scrape loading/dye transfer (SL/DT) assay as described in (18, 19).

2. In all experiments, hESC must be kept moisturized in buffers at all time to prevent dehydration.

3. Although GJIC can be modulated by Ca^{2+} in different cell types, we previously demonstrated that Ca^{2+} does not modify GJIC in hESC. Thus, for an easier handling of the cells, we suggest to perform SL/DT in the presence of Ca^{2+}.

4. Wash hESC colonies three times in a pre-warmed Ca^{2+}- and Mg^{2+}-PBS buffer (buffer 1, *see* **Note 10**).

5. Remove the plastic chambers of the slide, and if necessary, individualize each well with a wax pen.

6. Cut hESC colonies with a surgical blade followed by 5 min of incubation with Lucifer yellow (1 mg/ml) and rhodamine–dextran (1 mg/ml) diluted in buffer 1 (*see* **Note 11**).

7. After further washes with buffer 1, live colonies are viewed under a fluorescence microscope.

8. Control colonies incubated with both biochemical dyes without scraping should demonstrate no uptake or dye transfer of Lucifer yellow or rhodamine–dextran, confirming that the Lucifer yellow transfer is solely due to gap junction coupling rather than a leaky membrane.

9. If experiments are performed in order to assess the effect of specific acute treatments, the drugs used must be incubated at each step of the experiments (i.e., in buffers and Lucifer yellow/rhodamine–dextran solutions).

3.5 Chemical Closure of Gap Junctions

We previously found that a number of specific inhibitors and ligands can induce chemical closure of gap junction in hESC, such as PMA (1 µM, 60 min to activate protein kinase C), U0126 (60 µM, 60 min to inhibit MEK phosphorylation), glycyrrhetinic acid (α-GA, 10 µM, 24 h), and BMP-4 (10 ng/ml, 30 min). Using these gap junction blockers, one can readily study the effect of gap

junction closure on cell apoptosis, proliferation, pluripotency, and colony growth of hESC (*see* **Note 12**).

3.6 Cell Apoptosis Assay

1. Cell apoptosis is quantified using the in situ cell death detection kit by flow cytometry analysis.

2. hESC were cultured with or without α-GA (10 μM) for 24 h.

3. Collect floating apoptotic bodies in the media. Wash cells with PBS, and centrifuge at $1,300 \times g$ for 2 min to collect cells.

4. Harvest hESC colonies using dispase (10 min, 37 °C), and transfer hESC colonies to an Eppendorf tube.

5. Incubate with TrypLE Express (*see* **Note 13**) for 5 min at 37 °C. Wash cells with PBS, and centrifuge at $300 \times g$ for 2 min to collect cells. Gently pipette the cells up and down to break clumps to achieve single-cell suspensions. Mix the apoptotic bodies to the hESC samples. Use no more than 2×10^7 cells/ml.

6. Fix cells with 2 % PFA for 1 h at room temperature.

7. Wash fixed cells twice with PBS. Centrifuge at $1,300 \times g$ for 2 min to collect samples.

8. Permeabilize the samples with 0.1 % Triton X-100 in PBS for 2 min on ice.

9. Wash fixed cells twice with PBS. Centrifuge at $1,300 \times g$ to collect samples.

10. Incubate cells with 50 μl of "TUNEL reaction mixture" for 60 min at 37 °C. Negative controls are performed by incubating the cells in "label solution" only for 60 min in an incubator at 37 °C. An unstained sample should be prepared as a control to determine autofluorescence background.

11. Wash samples twice with PBS. Centrifuge at $1,300 \times g$ to collect samples.

12. Samples are analyzed by a flow cytometer. Negative control samples were used to set the gate and determine the background due to the secondary antibody; run the samples with voltages set so that the majority of the cells are in the left quadrant. All other subsequent samples should be run with the same voltage to ensure consistency. Collect at least 50,000 cells for analysis.

3.7 Cell Proliferation Assay

1. Cell proliferation in hESC can be quantified using the in situ cell proliferation kit by flow cytometry analysis.

2. Add BrdU (10 μM final) in the culture medium for 2 h in an incubator (37 °C) prior to harvesting the hESC. Negative control is performed with hESC without incubation with BrdU.

3. Harvest hESC by dispase treatment (10 min, 37 °C), and transfer cells to an Eppendorf tube.

4. Incubate with TrypLE Express (*see* **Note 13**) for 5 min at 37 °C. Wash cells with PBS, and centrifuge at 300 × *g* for 2 min to collect cells. Gently pipette the cells up and down to break clumps to achieve single-cell suspensions.

5. Rinse with PBS. Centrifuge at 300 × *g* for 2 min to collect samples.

6. Inject the cell suspension into 70 % ethanol and incubate for 30 min at −20 °C. Do not resuspend the cell pellet in 70 % ethanol to avoid cell aggregation.

7. Wash cells with PBS. Centrifuge at 300 × *g* for 2 min to collect samples.

8. Denature DNA with HCl 4 M for 10 min. Wash cells with PBS until pH >6.5 (~3–4 times). Centrifuge at 1,300 × *g* for 2 min to collect samples.

9. Incubate cells with 50 μl mouse anti-BrdU antibody conjugated with fluorescein for 45 min at 37 °C.

10. Rinse samples twice with PBS. Centrifuge at 1,300 × *g* for 2 min to collect samples.

11. Incubate with propidium iodide (20 μg/ml) for 15 min at room temperature. Do not wash away propidium iodide. Take samples directly to flow cytometry analysis.

12. Samples are analyzed by a flow cytometer. Negative control samples were used to set the gate and determine the background due to the antibody; run the samples with voltages set so that the majority of the cells are in the left lower quadrant. All other subsequent samples should be run with the same voltage to ensure consistency. Collect at least 50,000 cells for analysis.

3.8 Cell Pluripotency Assay

1. hESC pluripotency can be quantified by flow cytometry using stem cell markers TRA181 or TRA160.

2. Harvest hESC by dispase treatment (10 min, 37 °C), and transfer cells to an Eppendorf tube.

3. Incubate with TrypLE Express (*see* **Note 13**) for 5 min at 37 °C. Wash cells with PBS, and centrifuge at 300 × *g* for 2 min to collect cells. Gently pipette the cells up and down to break clumps to achieve single-cell suspensions.

4. Block with 1 % goat serum in hESC culture medium for 30 min on ice. *See* **Note 7**.

5. Incubate samples with TRA-1-60 or TRA-1-81 antibodies (1 μg/1 million cells, diluted in PBS with 1 % goat serum) for 30 min on ice. Negative control should be performed with hESC incubated with the appropriate concentration of mouse

IgM antibody. An unstained sample should be prepared as a control to determine autofluorescence background.

6. Wash samples twice with PBS. Centrifuge at $300 \times g$ for 2 min to collect samples.

7. Incubate with goat anti-mouse IgM antibody conjugated with Alexa Fluor® 488 (4 μg/ml, diluted in PBS with 1 % goat serum) for 30 min on ice.

8. Wash samples twice with PBS. Centrifuge at $300 \times g$ for 2 min to collect samples.

9. Samples were analyzed by a flow cytometer. Negative control samples were used to set the gate and determine the background due to the antibody; run the samples with voltages set so that the majority of the cells are in the left lower quadrant. All other subsequent samples should be run with the same voltage to ensure consistency. Collect at least 50,000 cells for analysis.

3.9 Colony Growth Assay

1. We previously found that the chemical inhibition of GJIC was accompanied by cell death and decrease of colony growth in serum-free medium, but not in culture medium containing serum.

2. Incubate hESC colonies for 5–7 days in serum-free medium in the presence or the absence of α-GA (10 μM), and change medium every 2 days.

3. Capture phase-contrast images of the morphology for at least 16 hESC colonies every day for 5–7 days.

4. Colony growth can be assessed by measuring hESC colony area using Analysis^B software. Alternatively, the colony diameter can be measured with reference to scale bar using Adobe Photoshop. The colony diameter is recorded as the average of the longest and shortest diameter of the colony.

3.10 Statistical Analysis

All experiments must be performed at least three times to ensure consistent results. Statistical analysis on raw data is performed using Graphpad Prism software. Different statistical tests may be used for the different experiments performed, such as the two-tailed *t*-test or one- and two-way ANOVA followed by Bonferroni or Tukey tests. Statistical significance is established at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

4   Notes

1. Formats of culture are reflective of what we found to be the easiest handling format to perform experimental work. These are only indicative and can be modified according to specific needs.

2. Dispase is a protease that cleaves adhesion molecules, thus allowing extraction of hESC from MEF and the plastic surface of the dish. hESC colonies can be readily sucked off using a pipette, leaving the MEF layer intact on the dish.

3. The system uses polyT magnetic beads to hybridize at high efficiency with the 3′ polyA tail of mRNA, thus yielding highly purified mRNA.

4. This technique allows for the study of the level of phosphorylation of Cx43 in hESC. Indeed, Cx43 can be present as unphosphorylated or phosphorylated once or twice. There is no strict correlation between the phosphorylation states of connexin proteins and the degree of functional coupling. Indeed, phosphorylation of Cx43 can influence GJIC both positively and negatively depending on the cell type ([20], [21]). The antibody used in this protocol recognizes three forms of Cx43 (unphosphorylated, phosphorylated once and twice).

5. Blocking the membrane with BSA rather than with dried milk gives a better resolution for the detection of phosphorylation.

6. If no detection is observed, the membrane can be incubated with 0.1 % (w/v) Ponceau S staining solution (0.1 % (w/v) Ponceau S, 5 % (v/v) acetic acid: mix 50 mg Ponceau S and 2.5 ml acetic acid, and bring to a final volume of 50 ml with distilled water) for 10–20 min with agitation to check for successful protein transfer. Membrane can then be washed in distilled water until clean.

7. The serum used for blocking should correspond to the species in which the secondary antibodies were raised. A combination of serum from different species can be used together.

8. hESC communicate through functional and opened gap junctions. The SL/DT assay allows for a quick, cheap, and reliable study of GJIC in cells. Controls: hESC in serum (GJIC are opened) and PMA (1 µM) for 60 min (GJIC are closed).

9. For an easier handling of cells during these experiments, it is advisable to cultivate hESC cells on 8-well chamber slides. Generally we use day-5 colonies as the colonies are large enough to handle and not yet started to spontaneously differentiate.

10. Ca^{2+}- and Mg^{2+}-free PBS buffer (buffer 2: 140 mM NaCl, 5.5 mM KCl, 10 mM glucose, 10 mM Hepes, 2 mM EGTA, pH 7.35) can be used to determine the effect of exogenous Ca^{2+} and Mg^{2+} in modulating gap junctions in hESC.

11. Due to its low molecular weight (522 Da), Lucifer yellow is able to diffuse from cell to cell through functional gap junctions. On the other hand, rhodamine–dextran (10 kDa) is too large to diffuse through gap junctions and thus serves as a

negative control. Time of incubation with Lucifer yellow can be modified depending on the size of the colonies.

12. Other techniques not used in the laboratory are available for the study of gap junctions in hESC. In particular, siRNA can be used to downregulate specific connexin proteins instead or in complement of a chemical inhibition of GJIC. Furthermore, other potent inhibitors of GJIC in different cell types, but not yet used in hESC, include heptanol, octanol, and halothane.

13. TrypLE Express is a trypsin-like enzyme used to dissociate cells into single cells. In our experience TrypLE Express is more gentle to hESC than trypsin.

Acknowledgments

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Immunofluorescence Microscopy and mRNA Analysis of Human Embryonic Stem Cells (hESCs) Including Primary Cilia Associated Signaling Pathways

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Abstract

This chapter describes the procedures for immunofluorescence microscopy (IFM) and quantitative PCR (qPCR) analyses of human embryonic stem cells (hESCs) grown specifically under feeder-free conditions. A detailed protocol is provided outlining the steps from initially growing the cells, passaging onto 16-well glass chambers, and continuing with the general IFM and qPCR anlysis. The techniques are illustrated with results on cellular localization of transcriptional factors and components of the Hedgehog, Wnt, PDGF, and TGFβ signaling pathways to primary cilia in stem cell maintenance and differentiation. Furthermore, a sample qPCR experiment is experimentally shown illustrating that these techniques can be important tools in answering basic questions about hESC biology.

Keywords: hESCs, Immunofluorescence microscopy, qPCR, Feeder-free, Cellular signaling, Primary cilia

1 Introduction

Stem cell research is a young but rapidly advancing research field whose potential benefits include the understanding of the molecular mechanisms that underlie a wide variety of human pathologies in the hopes of obtaining therapeutic tools in dealing with these conditions. Pluripotent embryonic stem cells (ESC) can give rise to all three germinal layers and can differentiate to form specific cell types depending on the conditions for culturing the cells. The use of human ESC (hESC) in regenerative medicine is still the ultimate goal for many research programs and in recent years this goal has been shown plausible for a number of diseases (1). Clinical trials using hESC derived retinal pigment epithelial cells in patients with degenerative eye illnesses have shown the first modest positive results (2).

However, the molecular and cellular functions leading to proliferation and differentiation still have yet to be characterized completely.

One set of proteins that are traditionally specific to hESC include the transcription factors, OCT3/4, SOX2, and NANOG. This triumvirate of proteins is important for stem cells whose interplay is thought to be responsible for the maintenance of the undifferentiated state. For example, it is thought that there are some 17,000 transcriptional targets for the OCT3/4 protein alone (3).Therefore, it is critical to examine how these transcription factors regulate pluripotency of stem cells, such as examining their spatiotemporal expression and location under different experimental conditions. This chapter describes how immunofluorescence microscopy (IFM) and "real-time quantitative reverse transcription PCR" (qPCR) analysis can be used to analyze important features of hESC cultured under feeder-free conditions. In this work we further examined the formation of primary cilia, and how these organelles are associated with multiple cellular signaling pathways that impinge on the mechanisms that control stem cell maintenance, differentiation, and self-renewal. Primary cilia are microtubule-based, nonmotile organelles that emerge as solitary organelles during growth arrest in most cell types of the human body (4) and coordinate signaling pathways in cell cycle control, survival, migration, and differentiation (5, 6). Consequently, defects in assembly or function of the primary cilia lead to a wide variety of developmental defects, diseases, and disorders, including polycystic kidney and liver disease, Bardet–Biedl syndrome, heterotaxia, pulmonary dysfunction, skeletal defects, retinal degeneration, obesity, diabetes, and cancer (7–10). Indeed, hESCs form primary cilia that coordinate Hedgehog and TGFβ signaling that control cell differentiation (11, 12). Therefore, studies on hESC primary cilia in feeder-free cultures may help understanding the complex signaling mechanisms that control early differentiation of hESCs.

2   Materials

2.1   Culture of hESCs Under Feeder-Free Conditions

1. 2× 16-well Lab-Tek Chamber Slide (Nunc, Cat #178599).

2. Petri dishes (35, 60 and 100 mm dishes, Nunc, Roskilde, Denmark, Cat # 153066, #150288 and #150350).

3. 35-mm dish with confluent layer of mitotically inactivated human foreskin fibroblasts (hFF) (Nunc, Cat # 153066).

4. Trypsin-resistant hESC line.

5. 0.1 % gelatin diluted in PBS.

6. Sterile PBS (prewarmed at 37 °C).

7. IMDM medium (IMDM (Gibco, Carlsbad, CA, USA, Cat # 21980-032) + 10 % FBS (Gibco Cat # 10500-064) + Penicillin/streptomycin (Gibco Cat # 15070-063) (prewarmed at 37 °C)).

8. hESC medium (Knockout DMEM (Gibco, Cat # 10829-018) + 15 % Knockout Serum replacement (Gibco, Cat # 10828-028) + bFGF (4 ng/ml) (R&D, Minneapolis, MN, USA, Cat # 234-FSE) + Pen/Strep + 1× nonessential amino acids (Gibco, Cat #11140-035) + 1× Glutamax (Gibco, Cat # 35050-038), 0.1 mM b-mercaptoethanol (Gibco, Cat # 31350-010)).

9. hFF medium (irradiated hFF grown in 175 cm^2 flask in a confluent layer (ca. 2×10^6 cells) with 25 ml IMDM medium harvested every 9 days and filter sterilized).

10. Conditioned medium (hFF medium: hESC medium = 1:1).

2.2 IFM Analysis on Cells Cultured in 16-Well Lab-Tek Chamber Slides

1. PBS (13.7 mM NaCl, 1.06 mM Na_2HPO_4, 0.21 mM KH_2PO_4, 0.11 mM K_2HPO_4 [pH 7.5]).

2. FBS (Invitrogen, Cat # 10108-165).

3. Bovine serum albumin (BSA; Affymetrix Cat # 9048-46-8).

4. Paraformaldehyde fixation buffer (4 % PFA in PBS).

5. Permeabilization buffer (0.1 % Triton X-100 in PBS and 1 % BSA).

6. 4 % BSA blocking buffer (4 % BSA in PBS).

7. Mounting medium 0.1 g *n*-propyl gallate (Sigma Aldrich, ST. Louis, USA, Cat #P3130) + 0.5 ml 10× PBS + 4.5 ml glycerol.

8. Tweezers.

9. Primary antibodies (Table 1).

10. Alexa Fluor 568 Donkey anti-mouse (1:600 PBS) (Invitrogen, Cat # A10037).

11. Alexa Fluor 488 Donkey anti-goat (1:600 PBS) (Invitrogen, Cat # A10055).

12. Alexa Fluor 488 Donkey anti-rabbit (1:600 PBS) (Invitrogen, Cat # A21206).

13. 4′,6-Diamidino-2-phenylindole (DAPI) (14.4 mM) (1:5,000 in 4 % BSA blocking buffer).

14. Nail polish.

15. Coverslips (22 × 74 mm, Nunc, Cat # 171080).

16. Whatman paper.

17. Kimwipes.

2.3 IFM Analysis on Cells Cultured on Coverslips

1. 100 ml BlueCap bottles.

2. Concentrated HCl.

3. 96 % EtOH.

4. 70 % EtOH.

Table 1
Primary antibodies used for IF protocol

Primary antibodies				
Name	**Species**	**Company**	**Catalog number**	**Dilution**
SOX2	Mouse	R & D Systems	MAB2018	1:100
SOX2	Goat	R & D Systems	AF2018	1:100
OCT3/4	Goat	Santa Cruz	sc-8629	1:300
OCT3/4	Rabbit	AbCam	AB19857	1:100
NANOG	Goat	R&D Systems	AF1997	1:100
α-Tubulin	Mouse	Sigma Aldrich	T3320	1:500
Acetylated α-tubulin	Mouse	Sigma Aldrich	T7451	1:3000
ARL13B	Rabbit	Santa Cruz	sc-8629	1:500
Dvl-1	Mouse	Santa Cruz	sc-8025	1:100
β-Catenin	Rabbit	Cell Signaling	1247S	1:200
phospho-β-Catenin (Ser$^{33/37}$/Thr^{41})	Rabbit	Cell Signaling	9561S	1:200
PDGFRα	Rabbit	Santa Cruz	SC12910-R	1:100
PDGFRβ	Rabbit	Santa Cruz	sc-432	1:100
Centrin	Goat	Santa Cruz	sc-8719	1:100
Pericentrin	Goat	Santa Cruz	sc-28145	1:100
Smo	Rabbit	MBL International	A2668	1:200
Ptc	Rabbit	Santa Cruz	sc-6149	1:200
TGFβ-RI	Goat	Abcam	ab121024	1:100
Phospho-TGFβ-RII (Tyr^{424})	Rabbit	Santa Cruz	sc-17007	1:100

5. PBS.

6. BSA.

7. Paraformaldehyde fixation buffer (4 % PFA in PBS).

8. Methanol fixation buffer (MeOH).

9. Permeabilization buffer (0.1 % Triton X-100 in PBS and 1 % BSA).

10. 2 % BSA blocking buffer (2 % BSA in PBS).

11. Mounting medium 0.1 g *n*-propyl gallate (Sigma Aldrich, St. Louis, USA, Cat #P3130) + 0.5 ml 10× PBS + 4.5 ml glycerol.

12. Tweezers.

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15. Alexa Fluor 488 Donkey anti-goat (1:600 PBS) (Invitrogen, Cat # A10055).

16. Alexa Fluor 488 Donkey anti-rabbit (1:600 PBS) (Invitrogen, Cat # A21206).

17. 4′,6-Diamidino-2-phenylindole (DAPI) (14.4 mM) (1:5,000 in 2 % BSA blocking buffer).

18. Nail polish.

19. Coverslips (round, 12 mm diameter, VWR, Cat # ECN 631-1577).

20. Microscope slides (76×26 mm, Thermo Scientific Cat #10144633B).

21. 100 mm Petri dishes.

22. Whatman paper.

23. Parafilm.

24. Kimwipes.

2.4 qPCR Analyses of hESC

2.4.1 Total RNA Extraction (Including DNase Treatment)

1. RNeasy Mini Kit (Qiagen, Cat # 74106).

2. QIAshredder homogenizer (Qiagen, Cat # 79656).

3. RNase-Free DNase Set (Qiagen # 79254).

4. Nuclease-free pipette tips with filter.

5. 70 % ethanol.

6. Microcentrifuge.

2.4.2 cDNA Synthesis (Reverse Transcription)

1. DNase-treated template RNA (from Section 3.3.1).

2. Nuclease-free pipette tips with filter.

3. 200 μl PCR tubes (Almeco, Cat # I1402-8100).

4. Nuclease-free water (Ambion, Cat # AM9915G).

5. OmniScript RT kit (Qiagen #205113).

6. Random hexamers (Qiagen #79236).

7. Thermal cycler (Thermo Scientific, Cat # TCA4848), Thermal block, or water bath.

2.4.3 qPCR Analysis

1. cDNA (from Section 3.3.2).

2. LightCycler instrument and software (Roche Applied Science, Basel, Switzerland).

3. LightCycler 480 SYBR Green I Master (Roche #04707516001).

4. PCR plate 96 wells (Roche #04729692001).

5. Seal plastic for plate (Roche #04729757001).

Table 2
Details for primers of six hESC genes (*NANOG, POU5F1, GABRB3, TDGF1, GDF3, DNMT3B*) and three reference genes (*ACTB, GAPDH, TBP*)

Gene symbol	Product name	Primer sequence (5′→3′)	Amplicon size (bp)
NANOG	Nanog	F: CAAAGGCAAACAACCCACTT R: CTGGATGTTCTGGGTCTGGT	426
POU5F1	Oct4	F: GACAACAATGAAAATCTTCAGGAGA R: TTCTGGCGCCGGTTACAGAACCA	218
GABRB3	GABA A receptor 3β	F: CAAGCTGTTGAAAGGCTACGA R: ACTTCGGAAACCATGTCGATG	108
TDGF1	Teratoma-derived growth factor 1 (Cripto)	F: AGCACAGTAAGGAGCTAAACA R: CAGTTCCGTCCGTAGAAGGAG	101
GDF3	Growth differentiation factor 3	F: GTACTTCGCTTTCTCCCAGAC R: GCCAATGTCAACTGTTCCCTT	131
DNMT3B	DNA methyltransferase 3β	F: AGCCACCTCTGACTACTG R: GACAAACAGCCATCTTCCA	149
ACTB	β-Actin	F: CCTGGCACCCAGCACAAT R: GGGCCGGACTCGTCATAC	144
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F: TCGGAGTCAACGGATTTGGT R: TTGCCATGGGTGGAATCATA	148
TBP	TATA-sequence-binding protein	F: CCCGAAACGCCGAATATAATC R: GACTGTTCTTCACTCTTGGCT	130

Primers were selected from PrimerBank [25] or designed with PerlPrimer [26]. All primer pairs have a melting temperature within the range of 60–62 °C
Abbreviations: *F* forward primer, *R* reverse primer, *bp* base pairs

6. Nuclease-free pipette tips with filter.

7. 10 mM primer working solutions for genes *NANOG, POU5F1* (i.e., the gene for OCT3/4), *GABRB3, TDGF1, GDF3, DNMT3B, TBP, GAPDH*, and *ACTB* (for primer details see Table 2).

3   Methods

Conventional culture techniques for most hESC lines rely on a feeder layer of mitotically inactivated mouse embryonic fibroblasts (MEFs) or human foreskin fibroblasts (hFFs) (for general hESC morphology in culture, please see Fig. 1a). Since feeder cells are a source of contamination and ambiguity problems when analyzing hESC for microscopy and/or RNA work, it is advantageous to grow hESC under feeder free conditions (Fig. 1a–d). However,
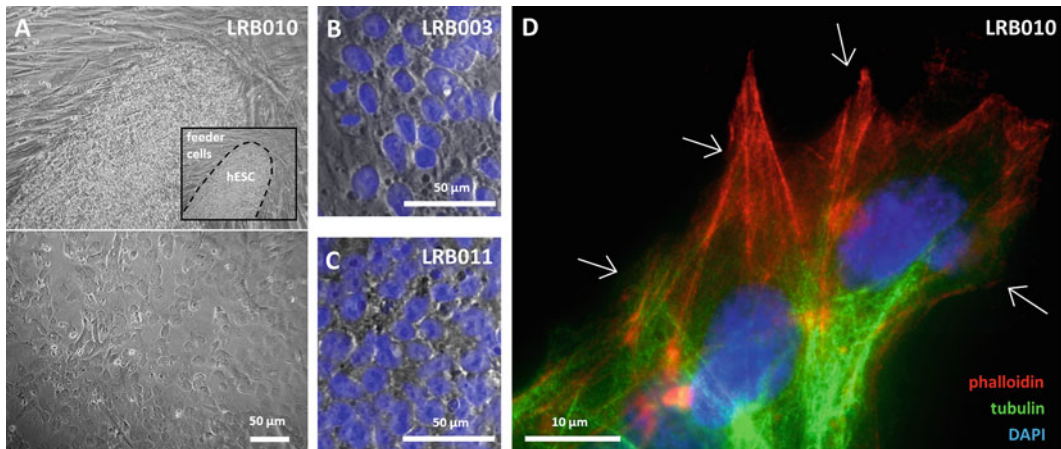
Fig. 1 Morphology of hESC grown under feeder-free conditions. (**a**) Light microscopy analysis of hESC line LRB010 grown in the presence (*upper panel*) and the absence of (*lower panel*) feeder cells (human foreskin fibroblasts, hff). (**b**, **c**) Morphology of hESC cell lines LRB003 and LRB011 grown in feeder-free cultures. Nuclei were stained with DAPI (*blue*). (**d**) IFM analysis of hESC (line LRB010) grown in feeder-free cultures and stained for F-actin (phalloidin, *red*), tubulin (anti-α-tubulin, *green*), and DNA (DAPI, nucleus, *blue*). The *arrows* indicate migrating cells at the border of the hESC colony

conditioned medium from feeder cell cultures may be required for hESC to thrive autonomously presumably because the medium contains key factors which help the hESCs to survive and proliferate (13). As such, when using trypsin-resistant hESC lines, it is important to have a supply of mitotically inactivated feeder layers from which to harvest the supernatant. This method of hESC cultured on gelatin may not be applied for continuing passage of cell lines. Colocalization studies using IFM can generate valuable information about stem cell biology, especially in terms of the differentiation modules involved. By using antibodies to the established stem cell markers (OCT3/4, SOX2, NANOG), hESC are easily detected in their undifferentiated state as evidenced by strong nuclear localization of the stem cell markers (Fig. 2a–c). The markers are absent in differentiated cells such as hFFs (Fig. 2d, e).

Recent research has shown that stem cells form primary cilia, which are associated with signaling pathways that regulate cell differentiation (12, 14–17). hESC in feeder-free cultures form primary cilia as visualized with IFM (Fig. 2f, g) using anti-acetylated α-tubulin, which marks stable microtubules in the axoneme of the cilium, and ARL13B, which is a cilia-enriched small GTPase of the Arf/Arl family that regulate ciliary structure and signaling, and is mutated in Joubert syndrome (8, 18). Interestingly, we here show localization of OCT3/4, SOX2, and NANOG to a subset of hESC primary cilia, indicating a functional relationship between primary cilia and coordination of the differentiation and/or self-renewal profile of stem cells (Fig. 2h, i). Indeed, a series
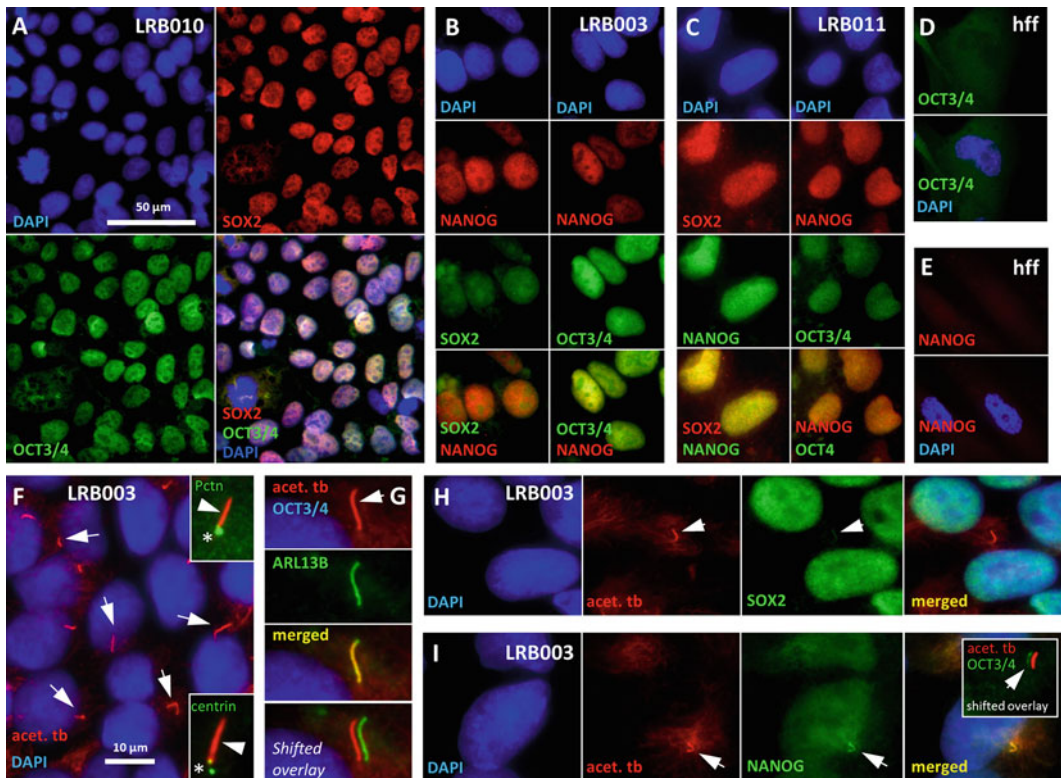
Fig. 2 IFM analysis on the localization of stem cell markers and primary cilia in hESC in feeder-free cultures. (**a**) Colocalization of SOX2 and OCT3/4 to the nucleus of hESC line LRB010. (**b**, **c**) Colocalization of SOX2, NANOG, and OCT3/4 to the nucleus of hESC lines LRB003 and LRB011. (**d**, **e**) Absence of OCT3/4 and NANOG in nuclei of feeder cells (hff: human foreskin fibroblasts). (**f**) presence of primary cilia (anti-acetylated a-tubulin, acet. tb, *arrows, red*) in confluent cultures of hESC line LRB003 that has entered growth arrest. Inserts: *Upper inset*: Anti-pericentrin (Pctn, *green*) marks the centrosome at the base (*asterisk*) of the primary cilium. *Lower inset*: The cilium (acet. tb, *red*) in LRB003 hESC specifically emerges from the centrosomal mother centriole (asterisk) marked with anti-centrin (centrin, *green*). From Kiprilov et al. (2008) with permission, courtesy of J. Cell Biology. (**g**) Localization of ARL13B (*green*) to the primary cilium (acet. tb, *arrow, red*) in an OCT3/4-positive cell of the hESC line LRB010. The *lowest panel* shows a shifted overlay between acet. tb and ARL13B. (**h**, **i**) Localization of SOX2 (**H**) and NANOG (**I**) (*green*) to the primary cilium (acet. tb, *arrows, red*) in hESC line LRB003. *Insert* (**i**): Shifted overlay showing localization of OCT3/4 (*green*) to the primary cilium acet. tb, *arrows, red* in hESC line LRB003. Nuclei were stained with DAPI (*blue*)

of receptors and their downstream signaling components, which are known to play essential roles in differentiation and/or proliferation in stem cells localize to hESC primary cilia (Fig. 3). hESC possess primary cilia that coordinate Hedgehog (Hh) signaling with ciliary Gli transcription factors as well as Patched (Ptc) and Smoothened (Smo) trafficking in and out of the cilium in response to Hh pathway activation (11) (Fig. 3a, b). Because Hh signaling facilitates the differentiation and proliferation of hESCs (19), these results support the notion that primary cilia play a critical role in modulating the signals for differentiation and/or proliferation in
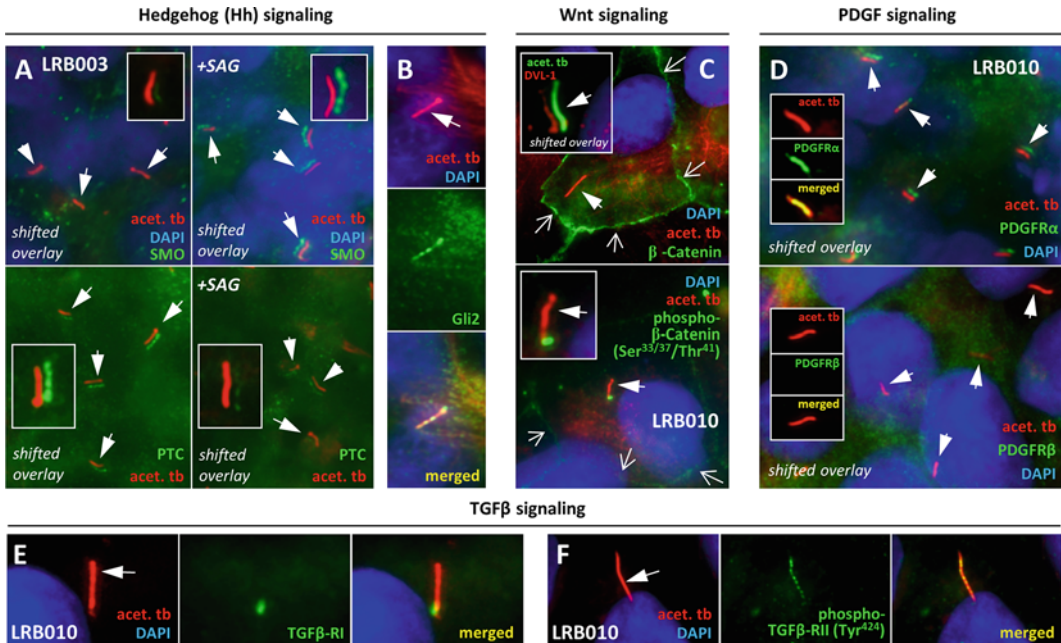
Fig. 3 IFM analysis on the localization of signaling components to the primary cilium (acet. tb, *bold arrows*) in hESC grown in feeder-free cultures. (**a**, **b**) Localization of Hedgehog signaling components to the primary cilium of hESC line LRB003. In the absence of Hh pathway stimulation, the Hedgehog receptor Patched (PTC, *green*) co-localizes with the cilium all along the ciliary membrane (**a**, *lower left panel*). *Red* and *green* channels are displaced (*shifted overlays with green fluorescence shifted to the right*) in the images to define co-localization more clearly. Upon stimulation with SAG, as part of the signaling cascade, PTC leaves the cilium (**a**, *lower right panel*) and Smoothened (SMO) enters to activate the hedgehog signaling cascade (**a**, *upper right panel*). (**b**) The Hh transcription factor, Gli2 (*green*), localizes to the primary cilium. From Kiprilov et al (2008), with permission, courtesy of J. Cell Biology. (**c**) Localization of Wnt signaling components to the primary cilium in hESC line LRB010. β-Catenin (*green*) predominantly localizes to the cell membrane (*upper panel*), whereas the phosphorylated form of β-catenin predominantly localizes to the base of the primary cilium (*lower panel*). *Insert upper panel*: Shifted overlay to show that Dishevelled-1 (DVL-1, *red*) localizes to the primary cilium (acet. tb, *bold arrow*, *green*). *Open arrows* mark the cell membrane. (**d**) Localization of PDGFR isoforms to the primary cilium in hESC line LRB010. Shifted overlay of PDGFRα (*green*) localizing to the primary cilium (acet. tb, *bold arrows, red*) (*upper panel*), and shifted overlay of PDGFRβ *(green)* not localizing to the primary cilium acet. tb, *bold arrows, red*) (*lower panel*). *Inserts* show merged images of PDGFR isoforms and the primary cilium. (**e**, **f**) Localization of TGFβ receptors (*green*) to the primary cilium (acet. tb, *bold arrows, red*) in hESC line LRB010. Nuclei are stained with DAPI (*blue*)

hESCs (Fig. 3a, b). Further, IFM of feeder-free cultures resulted in the localization of other signaling pathways in stem cells, including Wnt signaling that maintains the self-renewal, platelet-derived growth factor (PDGF)-receptor (PDGFR) signaling that helps maintaining hESCs in an undifferentiated state (20), and TGFβ signaling, which plays a critical role in stem cell lineage commitment, such as in cardiomyogenesis (12). Dishevelled-1 (Dvl-1) and the phosphorylated form of β-catenin in the Wnt pathway localize to the hESC primary cilium or at the base of the cilium (Fig. 3c),

whereas PDGFRα (but not PDGFRβ) localizes along the entire length of the primary cilium (Fig. 3d), similar to that observed in other cell types (6). Finally, TGFβ-RI and TGFβ-RII autophosphorylated at tyrosine in position 424 localize to the ciliary base and/or in a punctate pattern along the cilium (Fig. 3e, f). These observations favor the conclusion that the hESC primary cilium is an important sensory organelle both in maintenance of stem cell pluripotentiality and in regulation of early differentiation and proliferation.

Characterization of hESCs also includes RNA analysis in order to investigate the temporal expression of specific genes under various culture conditions. Importantly, the analysis of mRNA expression of stem cell markers has a few pitfalls, which need to be addressed in order to obtain valid results. Due to the presence of a substantial number of processed pseudogenes of *POU5F1* (*OCT3/4*) and *NANOG* in the human genome (21, 22), the extraction procedure of mRNA must include a complete degradation of genomic DNA. Processed pseudogenes are "transcriptional remnants," which have been incorporated into the genome by retrotransposition of the mRNA in early evolutionary history. Accordingly, they consist of a DNA sequence complementary to the same gene's mRNA (including untranslated regions). Therefore, the designing of primers with overlapping exon-intron boundaries will not evade the amplification of such pseudogenes, since the amplified genomic fragment will have the same amplicon size as the targeted gene sequence leading to false-positive results. In characterizing hESCs, we used six transcripts (*NANOG, POU5F1, GABRB3, TDGF1, GDF3*, and *DNMT3B*) thought to form a core group of ubiquitous hESC markers (23). These six transcripts were evaluated in our hESC lines and were shown to be highly expressed in undifferentiated hESC (Fig. 4). Furthermore, all six transcripts became significantly downregulated upon removal of FGF from the culture medium as determined by qPCR (Fig. 5). Thus, the use of this core group for hESC characterization on the mRNA level is validated. Primer pairs for all six transcripts (and three reference genes) are listed in Table 2.

3.1 Culture of hESCs

3.1.1 Day 1

1. Place a 16-well Lab-Tek Chamber Slide into a 100 mm Petri dish (helps prevent contamination) and remove cover from wells.

2. Add 200 µl gelatin to each well.

3. Place in 37 °C incubator overnight to allow gelatin to adhere to glass slide.

4. Similar procedures (1–3) are carried out for IFM analysis with cells cultured on coverslips in 35 mm Petri dishes (please see Section 3.2.2).
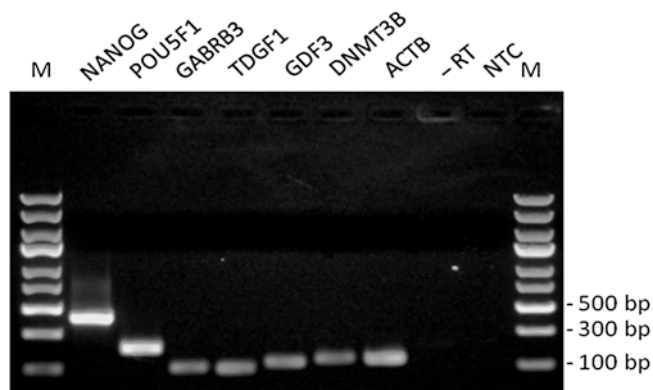
Fig. 4 Agarose gel electrophoresis of six proposed hESC markers and one reference gene (ACTB) evaluated in the LRB003 line [19]. The bands sizes correspond to the predicted amplicon sizes (see Table 2). The minus reverse transcriptase control was checked using *POU5F1* primers: The lack of a distinct band confirms the complete degradation of genomic DNA and hence *POU5F1* pseudogenes during the RNA extraction procedure. Abbreviations: *M* ladder, −*RT* minus reverse transcriptase control, *NTC* no template control, *bp* base pairs
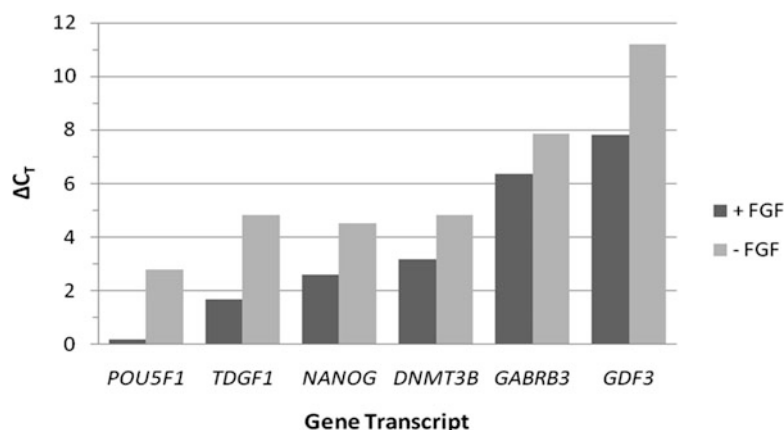
Fig. 5 ΔC_T values for a core group of six markers evaluated in the LRB010 hESC line. The smaller the ΔC_T value, the larger the amount of initial mRNA. ΔC_T values for each gene transcript were calculated by subtracting the geometric mean C_T value of three reference genes (*GAPDH*, *ACTB*, *TBP*) from the C_T (threshold cycle number) of each individual gene transcript. The increase in ΔC_T value for all six markers upon removal of FGF (mirroring incipient cell differentiation) reflects transcriptional downregulation (3- to 11-fold). A very similar pattern was observed for line LRB003, whereas the markers were completely absent in human foreskin fibroblasts (hFFs)

3.1.2 Day 2

1. Trypsinize a 35 mm dish grown with a near confluent layer of hESCs (which were plated on irradiated hFFs 5–7 days prior) by incubating in 1 ml 1× trypsin (prewarmed) for approximately 5 min. Pipette up and down gently to dissolve clumps (**Note 1**).

2. To stop the reaction, pipette the hESC-containing solution (1 ml) into 9 ml IMDM (discard any remaining clumps that have not dissolved).

3. Mix and centrifuge ($300 \times g$ for 5 min).

4. Resuspend pellet in 7 ml conditioned medium.

5. Remove the gelatin solution in each well and replace with 200 µl medium containing hESCs.

6. The remainder of hESC solution can be used for continuous passage of the cells by seeding them in culture dishes containing irradiated hFF cells or onto additional 16 wells.

7. Culture cells in a humidified incubator at 37 °C with an atmosphere of 6 % CO_2, 7 % O_2, and 87 % N_2 or alternatively in 5 % CO_2 in air.

8. Replace half the medium every 2 days until the cells form confluent layers.

9. Similar procedures (1–8) are carried out for IFM analysis with cells cultured on coverslips in 35 mm Petri dishes (please see Section 3.2.2).

10. For qPCR analysis cells are harvested either right after step 3 or can be seeded in 35 mm Petri dishes (placed in 60 mm Petri dishes to prevent contamination) that have been gelatin coated and cultured until the cells reach confluence. Harvest the cells by the same trypsin procedure as step 1–3 (please see Section 3.3).

3.2 IFM Analysis

3.2.1 IFM in Lab-Tek Chamber Slides

Day 1

1. Thaw the PFA fixation buffer from the freezer.

2. Aspirate medium from wells and wash with 200 µl PBS once. Be careful not to leave the wells dry.

3. Add 200 µl PFA fixation buffer and incubate for 20 min at room temperature.

4. Wash 3×5 min with PBS.

5. Add 200 µl permeabilization buffer and incubate for 20 min at room temperature.

6. Wash with PBS once and wait 5 min.

7. Repeat above step two more times.

8. Add 200 µl 4 % BSA blocking buffer for 45 min.

9. Make a humidified chamber by placing a H_2O-soaked Whatman paper in the Petri dish and place slide inside.

10. Remove the wells gently (they snap off with a little pressure from the sides) and remove the silicone rings with a tweezer by gently placing the tip of the tweezers under rings and pulling to other side slowly. If the rings tear apart, go back and repeat until all of the silicone is removed from the slides.

11. Use suction pipette tip (attached to vacuum) to dry the areas between wells. Place 25 μl PBS to avoid dehydrating in each of the 16 spots where the wells used to be. Make sure each well is distinct from the others so ultimately there will be 16 small pools of PBS per slide.

12. Dilute the primary antibodies in PBS (for complete list of antibodies, see Table 1).

13. Add 25–50 μl (less may be used as long as it covers the well) of the desired antibody solution to the appropriate wells. Make sure that the pattern is recorded.

14. Incubate at 4 °C overnight (alternatively, the primary incubation can be 60–90 min at room temperature).

Day 2

1. Wear gloves.

2. With vacuum suction, do five quick on/off washes per well with PBS.

3. Repeat with three quick on/off washes.

4. Turn off the lights and replace PBS with 25 μl of the correct secondary antibody mix to the appropriate wells (**Note 2**).

5. Let the slide incubate on the table with cover on top of Petri dish, under foil for 30–60 min.

6. Wash five times per well with PBS.

7. Replace PBS with 30 μl DAPI per well to stain nuclei (should stay on wells ca. 15 s). Suck off and replace with PBS. OBS: Only stain a few wells at the time, due to the short incubation time.

8. Wash each well again two times with PBS.

9. Repeat above two steps if you have many wells.

10. Dry the slide with the suctions and remove uneven surfaces (i.e., silicone pieces) on the glass (this is important so that there are no air bubbles left after the slide is sealed).

11. Place slide on Kimwipe. Add droplets of mounting medium (ca. 200 μl in total for 16 wells) on the coverslip (make sure that there are no air bubbles) and gently place a coverslip on top. Gently press on the coverslip to remove air bubbles. Afterwards, take end of the Kimwipe, fold over, and press firmly with the palm of your hand over the slide for 15 s to remove excess mounting medium/or remaining air bubbles (very critical to make a tight seal with the nail polish).

12. Seal the coverslip with nail polish around all four edges a few times and store it in a dark box at 4 °C for at least 20 min before viewing in microscope.

3.2.2 IFM with Cells Cultured on Coverslips in 35 mm Petri Dishes

Cleaning of Coverslips

1. Place 100 coverslips in a 100 ml BlueCap bottle.

2. Add 100 ml concentrated HCl until coverslips are covered.

3. Shake the bottle every 10 min for 1 h.

4. Discard HCl.

5. Rinse the coverslips in plenty of ddH_2O 10–15 times.

6. Rinse the coverslips in plenty of 96 % EtOH 10–15 times.

7. Replace with 70 % EtOH and store until use.

Preparation of Cells

1. Thaw the PFA fixation buffer from the freezer.

2. Remove medium from 35 mm Petri dishes and wash with 4 ml ice-cold PBS once. Be careful not to leave the coverslips dry.

3. Add 1.4 ml PFA fixation buffer to the 35 mm Petri dishes and incubate for 15 min at room temperature. For MeOH fixation, add 2 ml 20 °C MeOH and incubate for 15 min in the freezer.

4. Remove fixation buffers into waste containers, and then wash 2×5 min with PBS.

5. For PFA fixed cells, add 1.4 ml permeabilization solution to the 35 mm Petri dishes and incubate for 12 min. This step is not required for MeOH fixed cells.

6. Quench cells with 1.4 ml BSA blocking buffer for 30 min in the 35 mm Petri dishes and prepare humidity chambers consisting of H_2O-soaked Whatman paper overlaid with Parafilm in a 100 mm Petri dish.

7. Transfer coverslips with a tweezer to the humidity chambers with the cells facing upwards.

8. Add 100 µl of primary antibodies in BSA blocking buffer on top of each of the coverslips and incubate for 1.5 h.

9. Wash coverslips by suction 3×5 min in 100 µl BSA blocking buffer and add 100 µl fluorochrome-conjugated secondary antibodies in BSA blocking buffer on top of each of the coverslips (**Note 2**). Incubate for 45 min under foil.

10. For visualization of the actin cytoskeleton, F-actin can be stained with fluorochrome-conjugated phalloidin concomitantly with secondary antibody incubation.

11. After incubation, wash the coverslips once for 5 min in BSA blocking buffer and then incubate for 10 s with DAPI solution to stain DNA/nuclei followed by three washes, 5 min each, in PBS.

12. Place one or more microscope slides (precleaned with 96 % EtOH) on a Kimwipe.

13. Mount coverslips by placing them with tweezers, cells facing downwards, in a droplet of 20 µl mounting medium on the microscope slide.

14. After gently removing excess of mounting medium by pressing with the Kimwipe, seal the edges with nail polish, and store slides in a dark box at 4 °C for at least 20 min.

15. Visualize localization of antibodies and staining reagents with an epifluorescence or confocal microscope.

3.3 qPCR Analysis of hESCs

3.3.1 Total RNA Extraction (Including DNase Treatment)

1. Harvest cells, sediment, and completely aspirate cell-culture medium as described in Section 3.1.2, steps 1–3.

2. Wash one time in 10 ml PBS and centrifuge ($300 \times g$ for 5 min). Completely aspirate PBS and loosen the cell pellet thoroughly by flicking the tube.

3. Resuspend pellet in 350 µl of the RNeasy Mini Kit Lysis buffer with 1 % β-mercaptoethanol, prepared when used, and mix briefly by pipetting.

4. Pipette the lysate directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at full speed.

5. Follow Handbook for Qiagen RNeasy Kit at this point.

6. After step 5 in the Qiagen RNeasy handbook follow the step of DNase treatment.

7. After eluating RNA, aliquot 3 µl for determination of RNA yield and quality on a spectrophotometer.

8. Store immediately the remaining RNA at −80 °C. Under these storage conditions, no degradation of RNA is detectable for at least 1 year.

3.3.2 cDNA Synthesis

1. Thaw template RNA on ice (from Section 3.3.1). Thaw the primer solutions (Random hexamers), 10× Buffer RT, dNTP Mix, and RNase-free water at room temperature (15–25 °C). Store on ice immediately after thawing. Mix each solution by vortexing, and centrifuge briefly to collect residual liquid from the sides of the tubes (**Note 3**).

2. For each individual sample, a master mix should be made accordingly for a total of 20 µl:

   - 2 µl 10× Buffer RT
   - 2 µl dNTPMix
   - 2 µl Random hexamers
   - 1 µl Omniscript Reverse Trascriptase
   - Variable 2–13 µl Template RNA (up to 2 µg per reaction)
   - Variable 0–11 µl RNase-free water (add up for a total of 20 µl master mix)

3. If setting up more than one reverse-transcription reaction, make master mix without the template RNA and distribute the appropriate volume of master mix into individual reaction tubes (**Note 4**). Keep tubes on ice.

4. Add template RNA to the individual tubes containing the master mix. Mix thoroughly and carefully by vortexing for no more than 5 s. Centrifuge briefly to collect residual liquid from the walls of the tubes.

5. Incubate for 60 min at 37 °C preferably in a thermal cycler but alternatively in a thermal block or water bath.

6. Store cDNA at −20 °C or use immediately for PCR (**Note 5**).

3.3.3 qPCR

1. Thaw SYBR Green, template cDNA (from Section 3.3.2) (**Notes 5, 6**, and **7**), gene-specific primers (see Table 2), and nuclease-free water. Mix the individual solutions by inversion and brief spin (**Note 2**).

2. For every sample, the following are mixed and transferred to a well in the 96 well PCR plate:

   - 3 μl water
   - 1 μl Forward primer (10 μM)
   - 1 μl Reverse primer (10 μM)
   - 10 μl SYBR green
   - 5 μl cDNA template

3. Set up all reactions on ice and protect resolutions with SYBR green from light (**Note 8**).

4. It can be timesaving and give more consistent results if master mix is made either with the same cDNA template or primer sets.

5. The PCR plate is sealed with the sealing plastic and centrifuged at 2,800 × *g* for 3 min (remember counterweight) and placed in the LightCycler.

6. Run a PCR program on the LightCycler software in accordance with primer melting temperatures (Table 3) (**Notes 9** and **10**).

4   Notes

1. During trypsinization of stem cells, observe under the microscope when the cells de-adhere and be careful not to over-trypsinize. For each cell line and condition, times may vary accordingly.

Table 3
LightCycler program for real-time quantification of hESC transcripts and reference genes using SYBR Green dye detection chemistry

Program	Analysis mode	Temperature (°C)	Time	Ramp (°C/s)	Acquisition mode	Cycles
1. Preincubation	None	95	10 min	20	None	1
2. PCR	Quantification	95	10 s	20	None	45
		60*	20 s	20	None	
		72	30 s	20	Single	
3. Melting curve	Melting curve	95	5 s	20	None	1
		60	60 s	20	None	
		95		0.1	Cont	
4. Cooling	None	40	30 s	20	None	1

*Please *see* **Note 9**

2. When working with fluorophores conjugated to secondary antibodies or the SYBR mix, one has to work in the dark since they are light sensitive.

3. For cDNA synthesis, set up all reactions on ice and perform in an RNase-free environment as an RNA bench.

4. Include a Minus RT control with water instead of the RT enzyme in the cDNA synthesis step to confirm complete degradation of genomic DNA.

5. When performing qPCR, no more than 1/10 of the final PCR volume should derive from the finished reverse-transcription reaction (i.e., cDNA).

6. Before the qPCR reaction, all samples should be diluted to the same concentration using RNase-free water.

7. It is recommended to dilute the cDNA (e.g., $100\times$) before performing real-time PCR.

8. When performing the qPCR reaction include a no template control with water instead of cDNA template and run each sample in at least doublets and preferably triplets.

9. If primers are used with varying melting temperatures make sure to correct this in the quantification step of the LightCycler program (highlighted temperature in Table 3).

10. For subsequent data analysis, we calculate the geometric CT mean of the three reference genes TBP, GAPDH, and ACTB. Next, geometric reference CT mean is subtracted from the CT (threshold cycle) value for each target gene yielding a ΔCT value. The ΔCT values can be compared between separate cell populations or a fold change may be calculated using the comparative ΔCT method (24).

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Analysis of Intracellular Calcium Signaling in Human Embryonic Stem Cells

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Abstract

Measurement of changes in intracellular calcium concentration is one of the most common and useful tools for studying signal transduction pathways or cellular responses in basic research and drug screening purposes as well. Increasing number of such applications using human pluripotent stem cells and their derivatives requires development of calcium signal measurements for this special cell type. Here we describe a modified protocol for analysis of calcium signaling events in human embryonic stem cells, which can be used for other pluripotent cell types (such as iPSC) or their differentiated offspring as well.

Keywords: Human embryonic stem cell, Calcium signaling, Fluorescent calcium indicators, Genetically encoded calcium indicators, Confocal microscopy

1 Introduction

Calcium signals control diverse cellular processes, for example regulation of gene expression (1, 2), cell division or apoptosis (3), and activation of excitatory cell types (4). Analysis of these versatile patterns of calcium signals is essential for studying the mechanisms lying beyond the cellular functions. There are generally two widely used classes of calcium indicators, chemical calcium indicators (5, 6) and genetically encoded calcium indicators (GECIs) (7–9) for calcium imaging. Both types of indicators exhibit altered fluorescent properties according to changes in cytoplasmic calcium concentration. Selection of proper calcium indicator depends on the amplitude, frequency, and spatial and temporal properties of the Ca^{2+} transient; however, the "global" Ca^{2+} signals can be measured by most of the commonly used calcium indicators (10, 11). Human embryonic stem cell lines have normal genetic background and can differentiate toward various cell types which make them a favorable model system for studying calcium signals evoked by endogenous substrates and a wide range of drugs. At the same time there are only limited numbers of publications about calcium signals in human pluripotent stem cells (12, 13). This is partly due to the special feature of these cell types such as the formation of

three-dimensional clumps, presence of feeder cells, sensitivity of human PSCs to any enzymatic or mechanical cell separation, and heterogeneous shape and function of the PSCs even in a given clump (cell aggregate). Here we present a commonly usable method based on confocal microscopic system which eliminates the abovementioned problems and can be used for calcium signal analysis of hESC and hiPSC lines too.

2   Materials

2.1   Maintenance of hES Cells

All media were sterile filtered with 0.22 μm Steritop-GP (Millipore) vacuum filter units. All media and reagents were added at room temperature.

1. 0.1 % gelatin solution in water (*see* **Note 1**).

2. Feeder cell layer: Mouse embryonic fibroblast (MEF) cells isolated from embryos at day 13 (Millipore) and treated by Mitomycin C (Sigma).

3. MEF culturing medium: 90 % Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10 % Fetal Bovine Serum (Gibco), 1 mM Glutamax-I (Gibco). Store at 4 °C. The medium may be used within 2 weeks of preparation.

4. hES culturing medium: 80 % Knockout Dulbecco's Modified Eagle Medium (KoDMEM, Gibco) supplemented with 15 % Knockout Serum Replacement (Gibco), 1 mM Glutamax-I (Gibco), 0.1 mM beta-mercaptoethanol, 1 % nonessential amino acids, and 4 ng/mL human fibroblast growth factor (Invitrogen). Store at 4 °C. The medium may be used within 2 weeks of preparation.

5. hES cells were propagated on mitomycin C-treated MEF feeder layer by enzymatic dissociation with 0.05 % trypsin–EDTA (Invitrogen) and replated on fresh feeder layer every second day as described earlier (14).

6. For confocal microscopic measurements cells were seeded onto an eight-well Nunc Lab-Tek II Chambered Coverglass (Nalgene Nunc International).

2.2   Calcium Signal Analysis in hES Cells

1. Fluo4-AM: 1 mM solution in dimethyl sulfoxide (DMSO) (*see* **Note 2**).

2. Hank's balanced salt solution (HBSS) with calcium and magnesium, without phenol red (Invitrogen). Store at room temperature.

3. 100 mM adenosine-5′-triphosphate (ATP) solution (GE Life Sciences) (*see* **Note 3**).

4. Ionomycin (Invitrogen): 1 mM solution in DMSO (Invitrogen). Store at -20 °C. Ionomycin was used at a 5 μM final concentration for measurements.

5. EGTA: 100 mM solution in water, pH 7.4 (*see* **Note 4**).

3 Methods

3.1 Loading of hESCs with the Calcium Indicator, Fluo4-AM

1. Cover the eight-well chambered cover glass with 250 μL of 0.1 % gelatin solution for each well.

2. Store the coated chamber at room temperature under laminar flow cabinet for 2 h.

3. Remove the remaining gelatin solution, and seed mitomycin C-treated MEF cells onto the wells (4×10^4 cell/well in 250 μL MEF culturing medium). Prepare the MEF feeder layer at least 1 day before plating the hESCs (*see* **Note 5**).

4. Seed hESC cells (20–40 clumps/well) on to the mitomycin C-treated MEF feeder layer in 500 μL hES culturing medium 24–48 h prior to calcium signal measurement (*see* **Note 6**).

5. On the day of experiment dilute 0.3 μL of 1 mM Fluo4-AM in 300 μL FBS-free KoDMEM (1 μM final concentration). Keep this solution at room temperature in the dark (*see* **Note 7**).

6. Remove the hES culturing medium, add 200 μL diluted Fluo4-AM dye solution to one well, and keep the chamber in a CO_2 incubator for 15–30 min.

7. Wash the cells with HBSS before confocal imaging.

8. Finally, add 200 μL of HBSS solution to the cells and place the chamber onto the microscope (*see* **Note 8**).

3.2 Microscope Settings

1. All settings are adjusted to an Olympus IX-81 laser scanning confocal microscope using an Olympus PLAPO 20× objective. For Fluo4 imaging excite cells at 488 nm and collect emission data between 505 and 535 nm. Set image resolution to 512×512 or lower and scan speed to fast (about 1 s/scan) so that time resolution might be around 1 s. z-resolution should be 4 μm, and the duration of the measurement should be set to approx. 10 min (*see* **Notes 9** and **10**).

2. Time-lapse sequences were recorded with FluoView Tiempo (v4.3) time course software at room temperature.

3.3 Calcium Signal Analysis

1. Fix the chamber, and select an appropriate microscopic field (field of view) (*see* **Notes 11** and **12**).

2. Start scanning in XYT mode for about 1 min to determine baseline values.

3. Prepare 500 μM ATP solution: Add 0.5 μL of 100 mM ATP to 100 μL HBSS (*see* **Note 3**).

4. Add 50 μL of 500 μM ATP to the first well (final concentration 100 μM) very carefully (*see* **Note 13**), drop by drop, and continue scanning until calcium signal returns to baseline level (it takes a few minutes) (*see* **Note 14**).

5. The maximum and minimum levels of the calcium-dependent cellular fluorescence can be estimated in the presence of ionomycin and after the addition of excess EGTA to the medium, respectively. Prepare 30 μM ionomycin solution in HBSS: add 3 μL 1 mM ionomycin to 100 μL HBSS. Add 50 μL of this ionomycin solution to the cells very carefully (final concentration 5 μM).

6. After fluorescence intensity reaches its maximum use 33 μL 100 mM EGTA solution (final concentration 10 mM) to get the minimum fluorescence intensity level.

7. When fluorescence intensity reaches its minimum stop recording and save the experiment.

8. Repeat dye loading and calcium measurement in each well (*see* **Note 15**).

3.4 Data Analysis   Region of interest (ROI) was selected for each hESC clump (see Fig. 1) and was analyzed with the FluoView Tiempo (v4.3, Olympus, http://www.olympusmicro.com) software (*see* **Note 16**). Microsoft Excel software was used to analyze raw data. Relative fluorescence was calculated as percentage between maximal and minimal fluorescence:

$((F - F_{min}) \times 100)/(F - (F_{max} - F_{min}))$ (where F_{max} was the maximal fluorescence after addition of ionomycin and Fmin was the minimal fluorescence after addition of EGTA) (Fig. 1b, c and 2).

4   Notes

1. Weigh 0.25 g gelatin (type A, from porcine, Sigma) and transfer to the glass beaker. Add bi-distilled water to a volume of 200 mL. Mix, and heat gently. Make up to 250 mL with bi-distilled water after the solution has cleared up. Sterilize the solution in autoclave or by filtering.

2. Solve 50 μg Fluo4-AM (Molecular Probes) in 50 μL DMSO. Store at −20 °C. For calcium signal measurements Fluo4-AM was used at a 1 μM final concentration.

3. The ATP stock solution (100 mM) should be stored at −20 °C in aliquots. ATP solution was diluted freshly before experiment and stored at 4 °C. ATP was used at a 100 μM final concentration.
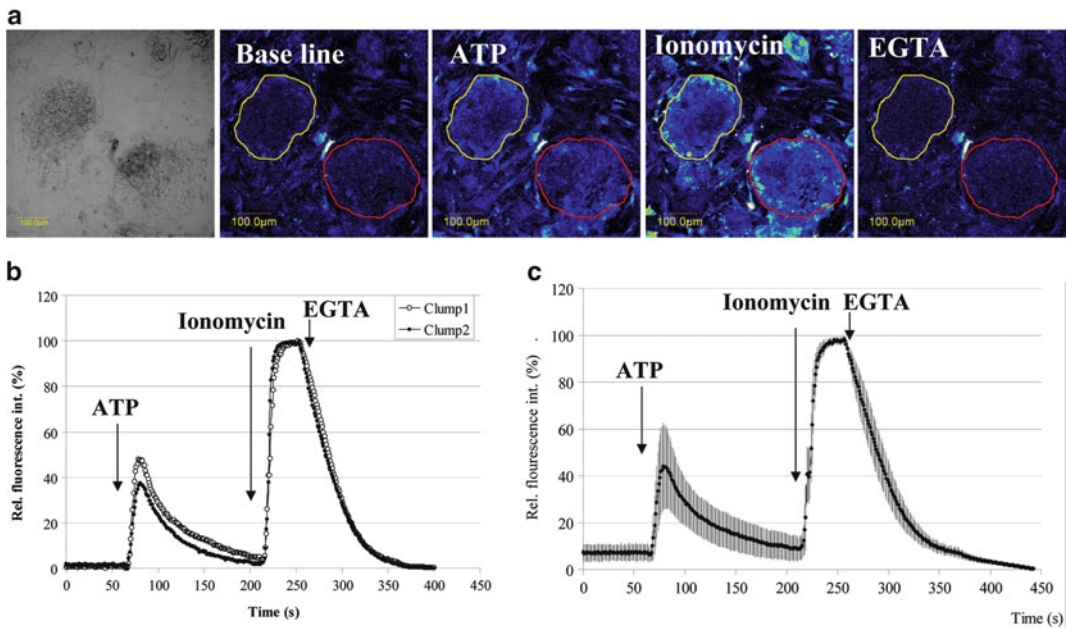
Fig. 1 Calcium signals in Fluo4-AM-loaded HUES 9 human embryonic stem cells. (**a**) Consecutive images were made after addition of ATP, ionomycin, and EGTA, respectively, as indicated in the pictures. The confocal images were artificially colored for better visualization of changes in calcium level. (**b**) Analysis of confocal microscopy imaging data was performed in the areas of hESC clumps assigned in (**a**) panel. (**c**) Statistical analysis of calcium signals measured on hESC clumps. Values represent the means $\pm$ S.D. of three independent experiments (13 clumps)
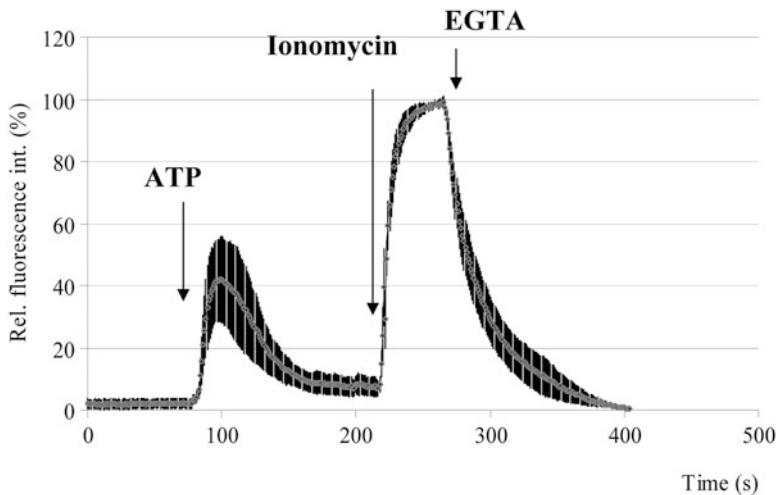
Fig. 2 Calcium signals in Fluo4-AM-loaded human induced pluripotent stem cells. Statistical analysis of calcium signals measured on hiPSC clumps. Values represent the means $\pm$ S.D. of three independent experiments (nine clumps)

4. Weigh 9.50875 g Titriplex VI (Merck) and transfer to the glass beaker. Add bi-distillated water to a volume of 250 mL. Mix, and adjust pH with 1.7 M Tris.

5. We found that culturing hESCs on an MEF layer is the most reliable method for calcium measurements. Cells might partially differentiate on a glass surface and shift more easily during treatments with ionomycin and EGTA without a feeder layer. Feeder cells can be simply excluded from the analysis according to morphological differences (Fig. 1a).

6. To avoid overgrowth of hESC clumps do not seed too many clumps into wells. The overgrown clumps can be partially differentiated on glass surface, and the loading of the fluorescent dye can be inhomogeneous within bigger clumps.

7. Diluted Fluo4-AM solution is not stable, and thus it is worth diluting it freshly each time.

8. Dye loading medium (KO-DMEM) and HBSS solution do not contain serum or serum replacement; thus, the hESC cells might starve during the experiment. Hence keep the cells in serum-deprived medium as short as possible.

9. The calcium signals can be studied by any other microscopic systems (for example wide-field fluorescent microscope); however, we found that the abovementioned confocal microscopic settings reflect calcium signals more accurately than others. Similar result has been found recently studying DT40 lymphocytes using GECI (15).

10. The calcium signals can be performed using GECIs, as well (16), utilizing the same confocal microscopic settings.

11. Fixing the chamber on the microscope is crucial; otherwise, any motion (e.g., when reagents are added) might fail the experiment.

12. The optimal field of view contains medium-size, compact hESC clumps (Fig. 1a).

13. Alternatively, calcium-free HBSS might be used to measure store-operated calcium entry (SOCE) (17).

14. The exact time of recording should be optimized for each calcium signal-inducing agent. Wait until the signal declines and a new equilibrium is reached.

15. We have used this method for studying calcium signals in induced pluripotent cells (18) as well (Fig. 2).

16. Images can be processed with ImageJ software as well.

Acknowledgments

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Genetic Manipulation of Human Embryonic Stem Cells

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Abstract

One of the great advantages of embryonic stem (ES) cells over other cell types is their accessibility to genetic manipulation. They can easily undergo genetic modifications while remaining pluripotent, and can be selectively propagated, allowing the clonal expansion of genetically altered cells in culture. Since the first isolation of ES cells in mice, many effective techniques have been developed for gene delivery and manipulation of ES cells. These include transfection, electroporation, and infection protocols, as well as different approaches for inserting, deleting, or changing the expression of genes. These methods proved to be extremely useful in mouse ES cells, for monitoring and directing differentiation, discovering unknown genes, and studying their function, and are now being extensively implemented in human ES cells (HESCs). This chapter describes the different approaches and methodologies that have been applied for the genetic manipulation of HESCs and their applications. Detailed protocols for generating clones of genetically modified HESCs by transfection, electroporation, and infection will be described, with special emphasis on the important technical details that are required for this purpose. All protocols are equally effective in human-induced pluripotent stem (iPS) cells.

Keywords: Human ES cells, Genetic manipulation, Transfection, Electroporation, Infection

1 Introduction

1.1 Genetic Modification Approaches and Their Potential Applications

There are basically two types of strategies that can be applied for inducing permanent changes in the DNA of HESCs. One approach depends on random integration of foreign DNA sequences into the genome while the other approach relies on targeted mutagenesis.

1.1.1 Random Integration of Foreign Sequences into the Genome

Random integration of foreign sequences into the genome is typically applied for overexpression of genes, or for the downregulation of endogenous genes in *trans* (knock-down). Overexpression is usually useful for constitutive or facultative expression of either cellular or foreign genes. It may also be applied for the introduction of reporter or selection genes, under the regulation of tissue-specific promoters. These procedures allow to label and track specific cell lineages following induced differentiation of human embryonic stem cells (HESCs) in culture. Moreover, they can be employed for the isolation of pure populations of specific cell types, by the use of selectable markers. The marker gene may either be a selectable reporter, such as green fluorescent protein (GFP),

resulting in the production of green glowing cells which can be selected for by fluorescent activated cell sorter (FACS), or a drug resistance gene (1–8). The ability to isolate pure populations of specific cell types and eliminate undifferentiated cells prior to transplantation has great importance in cell-based therapy; this is because transplantation of undifferentiated cells may lead to teratoma formation. Overexpression experiments may also be employed for directing the cell fate of differentiating ES cells in culture. This can be achieved by introducing master genes that play a dominant role in cell commitment, forcing the cells to differentiate into specific lineages that otherwise are rarely obtained among many other cell types in culture (9–12). Random integration of promoter-driven transgenes may also be employed for the generation of cell-based delivery systems by producing therapeutic agents at the site of damaged tissue. The use of ES-derived cells as therapeutic vectors has been previously shown to be feasible in mice, where grafting of ES-derived insulin secreting cells normalized glycemia in streptozotocin-induced diabetic mice (13).

Apart from tagging, selecting, and directing the differentiation of specific cell types, it is possible to inactivate endogenous genes to study their function. This can be achieved by downregulating the activity of particular genes in *trans* by overexpressing specific short hairpin RNA (shRNA) molecules. ShRNAs are short sequences of RNA that by forming hairpins silence target gene expression via RNA interference (RNAi) pathway. They are processed into small interfering RNAs (siRNAs) by the enzyme Dicer, and then paired with the target mRNA as they are incorporated into an RNA-induced silencing complex (RISC), leading to the degradation of the target mRNA. The great advantage of this system is that it provides a specific, long-lasting, gene silencing effect. This is why it is being considered as one of the most applicable tools for gene silencing in living organisms. Furthermore, since shRNAs operate in *trans* and are not involved in the modification of the targeted gene, it is relatively simple to apply and particularly efficient in achieving transient or conditional gene silencing effects. Expression of shRNA in HESCs is typically accomplished by transfection or through viral infection. Applications of this loss-of-function approach are now widely used not only to study developmental roles of specific genes in human, but also for their utility in modulating HESC differentiation in vitro (14, 15).

An additional use for the random integration approach can be the search of unknown genes whose pattern of expression suggests that they might have developmental importance. The identification of such genes is performed by the gene trap method, which is based on the random disruption of endogenous genes (reviewed by (16)). As opposed to targeted mutagenesis (*see* below), it involves the random insertion of a reporter gene that lacks essential regulatory elements into the genome. Because the expression of the reporter

gene is conditioned by the presence of an active endogenous regulatory element, it may serve to identify only transcribed sequences. Using this method, a large-scale gene disruption assay is possible, allowing the discovery of new genes and the creation of wide variety of mutations (17).

| 1.1.2 Targeted Mutagenesis | Targeted mutagenesis, or site-directed mutagenesis, is a procedure which involves the replacement of a specific sequence in the genome by a mutated copy through homologous recombination with a targeting vector. The targeting vector that contains the desired mutation and a selectable marker, flanked by sequences that are interchangeable with the genomic target, pairs with the wild-type chromosomal sequence and replaces it through homologous recombination. Targeted mutagenesis is most widely used technique for inactivating genes in ES cells. By targeting both alleles, using distinct selection markers, it is possible to create "loss-of-function" or so-called knockout phenotypes in ES cells that can be used for functional studies of specific genes. This technology has been well practiced in mice for gene function studies, in which genetically altered cells are introduced into wild-type embryos, resulting in the creation of germ-line transmitting chimeras (18). The genetically manipulated animals can be further mutated to generate animals that are homozygous for the desired mutation. The creation of HESCs with a null genotype for specific genes may have great importance for modeling human diseases, and for the study of crucial developmental genes that in their absence are embryonic lethal (19). Thus, these cells should be valuable for basic research studies, but more importantly for exploration of new gene therapy-based treatments and drug discovery. |

A very similar approach that relies on targeted mutagenesis involves the insertion of foreign sequences into the genome at desired loci. This strategy, termed knock-in, is commonly used to study the regulatory function of specific elements for example, by positioning a reporter gene under the regulation of a native gene. Therefore, it can be applied to follow the expression of a target gene in situ during ES cell differentiation and monitoring the expression of the endogenous genes, enabling to identify HESCs differentiated cell derivatives (20, 21).

It should be emphasized that both gene targeting approaches, knock-out and knock-in, depend on homologous recombination events however, the efficiencies of homologous recombination is extremely low (ranging from 1 in 10^6 to 1 in 10^7), limiting the routine use of these techniques in HESC manipulation until recently. Yet, as double strand breaks dramatically improve the rate of homologous recombination, it was hypothesized that by targeting double strand DNA breaks to specific sites in the genome one may significantly improve the efficiencies of targeted mutagenesis. Indeed, due to the recent advancements in the field of artificially

engineered nucleases, it has been possible to insert, replace, or remove specific DNA sequences from the genome of HESCs/iPS in a fairly uncomplicated procedure. This technology, termed genome editing, depends on the direction of unspecific DNA nucleases to desired sites in the genome, where they induce double strand DNA breaks and by that significantly enhance the rate of homologous recombination. There are by now three different types of engineered nucleases that can be applied for this purpose; zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and RNA-guided engineered nucleases (RGENs). All result in the elevation of gene targeting events through homologous recombination by at least 2–3 orders of magnitude relative to the conventional method by transient expression (22–27). Detailed description related to their composition, targetable sites specificities, off-target mutational rates, and complexity in design and preparation, which are beyond of the scope of this chapter, can be found in other excellent reviews (28, 29).

1.2   Methods for Genetic Manipulation

Several gene transfer techniques are now available for manipulating gene expression in HESCs. The latter include chemical-based (transfection), physical (electroporation), and viral-mediated (infection) techniques. No single transfection method will work for all HESC lines, and even within a lab, the method of choice may vary.

1.2.1   Transfection

Transfection is probably the most commonly used method for introducing transgenes into HESCs. It is straightforward, relatively easy to calibrate, provides a sufficient number of cells for clonal expansion, can be performed on adherent cell cultures, and allows the insertions of constructs of virtually unlimited size. This system is based on the use of carrier molecules that bind to foreign nucleic acids and introduce them into the cells through the plasma membrane. In general, the uptake of exogenous nucleic acids by the cell is thought to occur through endocytosis, or in the case of lipid-based reagents, through fusion of lipid vesicles to the plasma membrane. There are many factors that may influence transfection efficiency: phase of cell growth, number of passages, size and source of the transgene, vector type and size, and selection system. However, the most important factor is the transfection method. The first study to describe stable transfection in HESCs was based on the use of a commercially available reagent, ExGen 500, which is a linear polyethylenimine (PEI) molecule that has a high cationic charge density (1, 13). The unique property of this molecule is due to its ability to act as a "proton sponge," which buffers the endosomal pH, leading to endosome rupture and DNA release. This method routinely produces transient transfection rates of approx 10–20 % and stable transfection efficiencies of $1:10^{-5}$ to 10^{-6} (1). Since then, other chemical-based transfection methods have been found

to be equally effective. For example, Fugene6 (Roche) and Lipofectamine (Life Technologies) are commonly used by many labs. Both reagents are based on the presence of a positively charged cationic lipid compound that forms small unilamellar liposomes and are useful in obtaining transient and stable transfections in HESCs as well (15, 30). Usually, the cells are plated to 50–70 % confluence at the time of transfection. The plasmid DNA and lipid reagent are mixed in a tube, and only then administered to the cells as a DNA-lipid complex.

1.2.2 Electroporation

Electroporation is a method that employs the administration of short electrical impulses that create transient pores in the cell membrane, allowing foreign DNA to enter into the cells. Although efficient and most popular in mouse ES cells, this procedure gave poor results in HESCs, both in transient and stable transfection experiments. This is most probably due to the low survival rates of HESCs after the voltage shock. Zwaka and Thomson reported a protocol to increase the yield of electroporation 100-fold, thereby achieving an integration rate of approx $1:10^{-5}$ (21). This was performed by carrying out the procedure on cell clumps rather than on single cell suspension. In addition, electroporation was performed in standard cell culture media, which is a protein-rich solution, instead of PBS and altering the parameters of the protocol used in mouse ES cells. Using this method, 3–40 % homologous recombination events among resistant clones were reported, subject to vector properties (14). A substantial number of HESC clones obtained by homologous recombination have been created thus far using different constructs, demonstrating the feasibility of this technique for site-directed mutagenesis in HESCs.

1.2.3 Infection

Unlike in all nonviral-mediated methods (transfection and electroporation), gene manipulation by viral infection can produce a very high percentage of modified cells. To date, genetic manipulation of HESCs by viral infection has been reported by several groups using adeno- as well as Baculovirus and lenti-viral vectors (26, 31–33). Infection studies with RNA and DNA viruses have demonstrated that these viral vectors have two distinct advantages over other systems: high efficiency of DNA transfer and single-copy integrations. However, integration occurs randomly and cannot be targeted to a specific site in the genome. Yet, because of its high efficiency, this method could prove useful for bypassing the need for selection and time consuming clonal expansion, as well as for experiments that aim for random insertion mutagenesis or gene trap.

Lentiviral-based vectors offer an attractive system for efficient gene delivery into HESCs. Lentiviral vectors (LVVs) can transduce both dividing and nondividing cells and were shown to drive gene expression efficiently in various types of 'stem' cells. Gene delivery into HESCs by vectors derived from lentiviruses has the following

advantages: (1) lentiviral vectors efficiently transduce HESCs; (2) they integrate into the host-cell genome, thus promoting stable transgene expression; (3) transgene expression is not significantly silenced in undifferentiated HESCs as well as following differentiation; and (4) transduced HESCs retain their self-renewal and pluripotent potential. To improve vector biosafety and performance, all pathogenic coding sequences were deleted, resulting in a replication-defective vector. In addition, the proteins necessary for the early steps of viral infection (entering into the host cell, reverse transcription, and integration) were provided in *trans* by two additional plasmids: a packaging plasmid expressing the gag, pol, and rev genes, and an envelope plasmid expressing a heterologous envelope glycoprotein of the vesicular stomatitis virus (VSV-G). Third, a large deletion was introduced to abolish the viral promoter/enhancer activity. These steps resulted in a vector that could only undergo one round of infection and integration, a process termed transduction. Moreover, they minimized the risk of generation of wild-type HIV-1 by recombination.

Random chromosome integration of viral vectors poses the risk of insertional mutagenesis, oncogene activation, and cellular transformation. In addition, lentiviral vectors may not be suitable for transient transgene expression. Viral vectors derived from adenovirus and adeno-associated virus (AAV) have a much lower risk of insertional mutagenesis and have been tested in HESCs, but their transduction efficiencies were less satisfactory (26). The insect baculovirus *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV)-based vectors have also been introduced as a type of delivery vehicle for transgene expression in mammalian cells (34). The virus can enter mammalian cells but does not replicate, and it is unable to recombine with preexisting viral genetic materials in mammalian cells. One significant advantage of using baculovirus AcMNPV as a gene delivery vector is the large cloning capacity to accommodate up to 30 kilobases (kb) of DNA insert, which can be used to deliver a large functional gene or multiple genes from a single vector.

1.2.4 Short- vs.
Long-Term Expression

Gene transfer experiments can be subdivided into short-term (transient) and long-term (stable) expression systems. In transient expression, the foreign DNA is introduced into the cells and its expression is examined within 1–2 days. The advantage of this assay is its simplicity and rapidity. Furthermore, because the foreign DNA remains episomal, there are no problems associated with site of integration and the copy number of the transgene. Yet, it does not allow conducting experiments over long periods. Moreover, transfection efficiency usually does not exceed 20 %. For short-term induction, efficient transient expression can be

achieved through the insertion of supercoiled plasmid DNA rather than the linear form. Transient expression in HESCs usually peaks roughly 48 h after transfection, and frequently results in high expression levels attributed to the high copy number of plasmid DNA molecules that occupy the cell. During long-term assays, one isolates a clone of HESCs that has stably integrated the foreign DNA into its chromosomal genome. The major advantage of this method is the ability to isolate stable ES cell lines that have been genetically modified and can be grown indefinitely in culture. In this type of experiment, it is important to linearize the vector, leading to greater integration and targeting efficiencies. When the target gene is nonselectable, one must introduce also a positive selection marker under the regulation of a strong constitutive promoter. This can be performed either by cotransfecting the selectable marker on a separate vector, or as is frequently done, by fusing the selectable marker to the targeting vector. Selection should not be carried out immediately after transfection but at least 24 h later, giving the cells time to recover, integrate the foreign DNA and express the resistance conferring gene.

2   Materials

2.1   Tissue Culture (See Notes 1 and 2)

1. Knockout DMEM-optimized Dulbecco's modified Eagle's medium for ES cells (Life Technologies; cat. no. 10829-018).
2. DMEM 4.5 g/L glucose (Sigma, Dorset, UK; cat. no. D5796).
3. 1 M β-mercaptoethanol (Sigma; cat. no. M7522).
4. Nonessential amino acids 100× stock (Biological Industries, Kibutz Beit-Haemek, Israel; cat. no. 01-340-1B).
5. Insulin-transferrin-selenium 100× (Life Technologies; cat. no. 41400-045).
6. Bovine serum albumin (Sigma; cat. no. A-4919).
7. Mitomycin C (Sigma; cat. no. M-0503).
8. 0.1 % gelatin (Sigma; cat. no. G-1890).
9. Collagenase type V (Life Technologies; cat. no. 17104-019).
10. Hygromycin B (Sigma; cat. no. H-3274).
11. 6-thioguanine (Sigma; cat. no. A-4660).
12. Opti-MEM I (Life Technologies; cat. no. 31985-047)
13. TransIT-LT1 transfection reagent (Mirus).
14. TrypLE Select (Life Technologies, cat. no. 12563-011)
15. KnockOut SR-serum-free formulation (Life Technologies; cat. no. 10828-028).
16. Fetal calf serum (Biological Industries).

17. L-glutamine 100× stock (200 mM/L, Biological Industries; cat. no. 03-020-1).

18. Penicillin (10,000 U/mL) and streptomycin (10 mg/mL) 100× stock (Biological Industries; cat. no. 03 031-1B).

19. Human basic fibroblast growth factor (bFGF) stock solution (2 ng/μL) (human recombinant; Life Technologies; cat. no. 13256029).

20. Trypsin-EDTA: 0.25 % trypsin and 0.05 % EDTA (Biological Industries; cat. no. 03-052-1).

21. G418 (Geneticin; Sigma; cat. no. G-9516).

22. Hexadimethrine Bromide (polybrene) (Sigma H9268-5G).

23. Puromycin (Sigma; cat. no. P8833).

24. ROCK inhibitor Y-27632 (ATCC; cat. no. ACS3030)

25. Dimethylsulfoxide (DMSO; Sigma; cat. no. D-2650).

26. 10 mM β-mercaptoethanol: dilute 1:100 in PBS, filter, sterilize, and store at 4 °C.

27. 50× Mitomycin-C: dissolve 2 mg in 4 mL MEF medium, store in 4 °C.

28. bFGF solution: add 10 μg of bFGF solution to 5 mL of filter-sterilized 0.1 % bovine serum albumin dissolved in 1× PBS (with Ca^{2+}/Mg^{2+}), to give a final concentration of 2 μg/mL, store 1-mL aliquots in −20 °C.

29. 0.1 % gelatin solution: add 0.1 g of gelatin into a bottle containing 100-mL distilled water and autoclave immediately. The gelatin is dissolved while boiling in the autoclave, store at 4 °C.

30. MEF media: add to a 500-mL bottle of DMEM (high glucose and L-glutamine) 50-mL fetal calf serum, 2.5 mL penicillin/streptomycin, 5 mL Glutamine.

31. HESC medium: add to a 500-mL bottle of Knockout DMEM: 75 mL KnockOut SR, 6 mL nonessential amino acids, 6 mL glutamine (2 mM), 3 mL insulin-transferrin- selenium, 60 μL β-mercaptoethanol (0.1 mM), 3 mL penicillin/streptomycin, and 1 mL bFGF. ES media should be protected from light (*see* **Note 3**), and stored in 4 °C up to 1 month.

32. Collagenase solution: dissolve 10 mg of Collagenase type V in 5 mL serum-free DMEM (2 μM/1 mL working solution) and filter through a 0.2 μm filter under sterile conditions. Prepare fresh once a week. Store in 4 °C.

33. Freezing medium: add 1 mL of DMSO and 1 μL of ROCK inhibitor Y-27632 (10 μM stock) to 9 mL of appropriate HESC media. Media should be prepared fresh.

34. Leishman's stain (BDH, Poole, England) in 100 % methanol.

35. 293T cells medium: add to a 500-mL bottle of DMEM (high glucose and L-glutamine) 50-mL fetal calf serum, 2.5 mL penicillin/streptomycin, 5 mL Glutamine.

2.1.1 Equipment and Supplies for Tissue Culture

1. Laminar flow hood.

2. Humidified incubator set at 37 °C and 5 % CO_2.

3. Phase contrast microscope (objective range from $10\times$ to $40\times$).

4. Liquid nitrogen storage tank.

5. Refrigerator (4 °C) and freezers (-20 °C, -70 °C).

6. 37 °C water bath.

7. Electroporator (Biorad, Gene Pulser II System).

8. Swing-out centrifuge for conical tubes (15- and 50-mL).

9. Cell counter.

10. Gene pulser cuvette 0.4 cm electrode gap (Bio-rad cat#165-2088).

11. Pipetmen (2, 10, 20, 200, and 1,000 μL) designated for tissue culture use only.

12. Sterile forceps and scissors for dissecting mouse embryos.

13. Falcon tissue culture plates (100 × 20 mm) and 6-, 12-, and 24-multiwell trays (Falcon, Bedford, MA; cat. no. 353047, 353047, 353043, 353046).

14. Falcon 15-mL and 50-mL (Falcon; cat. no. 352097, 352098) polypropylene conical tubes.

15. Cryo vials (1.8-mL CryTube; Nunc, Roskilde, Denmark; cat. no. 363401).

16. Plastic pipets (1-, 2-, 5-, and 10-mL).

17. Tips for 2-, 10-, 20-, 200-, and 1,000-μL pipetmen.

18. Eppendorf tubes (1.5-mL).

19. Disposable filter unit FP 30/0.45 CA-S, 0.45 μm and 0.2 μm, cellulose acetate sterile (Whatman cat. no. 10462100 and 10462200, respectively).

20. Syringes sterile 20 mL.

2.2 Transfection

1. TransIT-LT1 Transfection reagent (Mirus).

2. Humidified incubator set at 37 °C, 5 % CO_2.

3. Tips for 2-, 10-, 20-, 200-, and 1,000-μL pipetmen.

4. 15-mL Falcon tubes.

5. Sterile eppendorf tubes (1.5-mL).

6. Opti-MEM I Reduced-Serum Medium (Life Technologies).

2.3 Infection	1. DMEM growth medium with 10 % FCS, and Glutamine (1 mg/mL), without penicillin/streptomycin.
	2. 27 µL of TransIT-LT1 (Mirus).
	3. Hexadimethrine Bromide (polybrene) 5 µL (8 mg/mL).
	4. Humidified incubator set at 34 °C, 3 % CO_2.
	5. Tips for 2-, 10-, 20-, 200-, and 1,000-µL pipettes.
	6. 15-mL tubes.
	7. Eppendorf tubes (1.5-mL).
	8. Tissue culture plates
2.4 Colony Picking	1. HESC medium (*see* Section 2.1, **item 26**).
	2. G418 (200 µg/mL).
	3. Puromycin (0.5–1 µg/mL).
	4. Hygromycin (100 µg/mL).
	5. 6-Thioguanine (1 µg/mL).
	6. 6-, 12-, and 24-well Falcon tissue culture plates (*see* Section 2.1.1, **item 11**).
	7. Mouth apparatus consisting of an aspirator mouthpiece, tubing, and Pasteur pipette pulled on flame for collecting single colonies (*see* **Note 4**).

3   Methods

3.1   Tissue Culture (See Notes 5 and 6)

3.1.1   MEFs

The special growth conditions that are required for supporting undifferentiated growth of HESCs in culture rely mostly on the presence of inactivated fibroblasts, serving as a feeder layer. The feeder layer sustains undifferentiated growth by secreting unknown growth factors, and by serving as a growth matrix that allows the cells to adhere and grow as monolayer culture. So far, primary mouse embryonic fibroblasts (MEFs) were the most commonly used in the propagation and derivation of HESCs. However, STO cells (34), fetal muscle (35), foreskin fibroblasts (36, 37), and marrow cells (38) were also reported to be equally effective in supporting undifferentiated growth. The feeders are prepared only from early passage MEFs (up to passage 5). Their mitotic inactivation is carried out by the treatment with mitomycin-C (39), but can also be achieved through irradiation (40). Normally we prepare MEFs from 13.5-days-old ion cyclotron resonance (ICR) embryos. However, inactivated primary fibroblasts are required not only for routine maintenance of ES cells in culture, but also for stable transfection experiments, where drug selection is applied. Therefore, it is a prerequisite that feeder cells be resistant to the drug employed. For this purpose,

one must separately prepare MEFs from different strains of mice that bear resistance to the desired drug or alternatively, use feeders that carry multidrug-resistant genes by intercrossing between different strains. For instance, the transgenic strain of mice DR-4, expresses four different drug-selected genes and can be used for the production of MEFs, which confer resistance to G418, puromycin, Hygromycin, and 6-thioguanine drugs (41). The DR-4 strain, therefore, represents a suitable and an economical donor for the production of drug-resistant MEFs, and is especially advantageous for gene targeting experiments, which normally involve sequential selection for multidrug-resistant markers. There may be a significant variability between various batches of MEFs, with respect to their capacity for supporting undifferentiated proliferation of HESCs. To overcome this problem, the competence of different batches of MEFs to support undifferentiated growth can be assessed by testing their ability to maintain undifferentiated proliferation of mouse or primate ES cell lines before their use.

Isolation of MEFS

1. Coat plates with 0.1 % gelatin by incubation for 10 min at room temperature.

2. Collect 13.5-days-old fetuses from pregnant mice using sterile equipment: sacrifice pregnant mice and dissect the embryos by removing the uterus and transferring it into a sterile PBS-containing Petri dish.

3. Rinse twice in PBS and relocate all work to laminar flow hood.

4. Using sterile tweezers and scissors, remove the fetuses from the uterus, separate them from extraembryonic tissues (amniotic and yolk sacs) and transfer them to a clean Petri dish with PBS.

5. Count the number of collected fetuses and prepare, for later use, 1×10-cm gelatin-coated tissue culture dish for every three fetuses.

6. Remove head and internal parts (liver, heart, kidney, lung, and intestine) with sterile tweezers under a stereomicroscope.

7. Cut the remaining tissues into small pieces in a minimal volume of PBS (1–2 mL) and transfer into a sterile 50-mL Falcon tube.

8. Disaggregate the cell clumps obtained by passing them through a 5-mL syringe with an 18-gauge needle, no more than 10 times.

9. Add MEF media to reach 10 mL per three embryos, distribute cell suspension evenly into 10-cm tissue culture dishes and incubate.

10. Change media the following day. When plates are confluent (2–3 days after dissection) split 1:3 by trypsinization.

11. Change media (10 mL) every 2 days. When cell density reaches confluence, trypsinize the cells and freeze each 10-cm plate in one cryovial, store in liquid nitrogen.

Mitomycin-C Inactivation of MEFs

1. Thaw contents of one cryotube into $3\times$ 10-cm culture dishes.

2. Grow the cells to confluence by changing the media every other day.

3. Further propagate the cells by splitting them twice at a 1:3 dilution (sums to 27 plates).

4. To inactivate the cells, add 40 μL of mitomycin-C stock solution (1 mg/mL) to 5 mL culture media (final concentration of 8 μg/mL) and incubate at 37 °C, 5 % CO_2, for 3 h.

5. Aspirate the mitomycin C-containing medium and wash the plates twice with 6 mL PBS.

6. Tripsinize cells by adding 1 mL of trypsin-EDTA and incubate at 37 °C, 5 % CO_2, for 5 min.

7. Add 5 mL medium and suspend the cells by vigorous pipetting.

8. Collect cell suspension into a 50-mL Falcon tube.

9. Centrifuge mitomycin-treated cell pool at $1{,}000 \times g$ for 5 min.

10. Aspirate supernatant and add fresh medium to reach a final cell concentration of 4×10^6 cells/10-cm dish. Feeder plates can be stored in the incubator for 3–4 days, but should be examined under the microscope before use.

11. It is possible to freeze mitomycin-C treated MEFs and keep them for later use. For this purpose freeze 1.5–7×10^6 cells in each cryotube and later thaw and plate to give 1–$5 \times$ 10-cm dishes, respectively.

3.1.2 Maintenance of HESCs and Genetically Modified Clones

The maintenance of HESCs in culture relies on the continuous and selective propagation of undifferentiated cells. Controlling culture conditions and minimizing the effect of spontaneous differentiation, which constantly occurs, can achieve this. When passing the cells, care must be taken so that the cell number will not drop below a certain density, because this increases their tendency to differentiate, possibly from a lack of autocrine signaling. The differentiation status of the cultures should be followed daily by observation through a phase-contrast microscope. Undifferentiated colonies are easily recognized by their typical appearance, which includes small and equal-sized cells that are defined by a discrete border, pronounced nucleus and clear cellular boundaries. As differentiation begins, the cells at the periphery of the colonies lose their typical morphology. At that stage, splitting must be performed.

Subculture of HESCs	1. Aspirate medium from plate and rinse with PBS.
	2. Replace with 1 mL serum-free DMEM containing collagenase type IV (2 mg/mL) per well.
	3. Incubate at 37 °C in a 5 % CO_2 atmosphere for 40–60 min.
	4. Add 1 mL growth medium and suspend the cells by gently pipetting.
	5. Using a 2 mL or a 5 mL pipette, collect cell suspension from plate into a conical tube making sure to break up cell clumps by pipetting (colonies should be reduced to approximately 5–20 cells) (*see* **Note** 7).
	6. Let cell clumps sink to the bottom of the tube for 10–15 min.
	7. Remove medium with collagenase carefully, and resuspend with fresh media by splitting 1:2–1:3.
	8. Plate on mitotically inactivated feeders prepared the previous day.
	9. After 48 h, replace medium with fresh hESC medium.
Freezing HESCs	1. Collect HESCs and pellet them, as described in Section " Subculture of HESCs", **steps 1–4**.
	2. Resuspend cells in an appropriate amount of growth media supplemented with 10 % DMSO and 1 μL/1 mL ROCK inhibitor (10 μM stock) (*see* **Note** 8).
	3. Mix the cells are gently by pipetting up and down and place in a properly marked cryotube.
	4. Store at −70 °C in a low temperature vial container filled with isopropanol for at least 1 day.
	5. For long-term storage, vials must be kept in liquid nitrogen.
Thawing HESCS (*See* **Note 9**)	1. Incubate the frozen cryovial in a 37 °C water bath until it is completely thawed.
	2. Transfer and resuspend the cells with 5 mL growth media in a conical tube.
	3. Pellet the cells by centrifugation at 1,000 × *g* for 5 min.
	4. Resuspend again in an appropriate amount of fresh media with 1 μL/1 mL of ROCK inhibitor (10 μM stock) (*see* **Note** 8).
	5. Plate cells and incubate overnight.
Mouse ES Cells Clonal Assay to Test Competence and Quality of KO-Serum Batch	Batch-to-batch variability in the competence of the KO-serum replacer to support undifferentiated proliferation may be remarkable. Clonal assays with mouse ES cells may be used to test the quality of the serum substitute batch before its use. An established culture of mouse ES cells is used as previously described (42) and all medium components should be those that will be used to culture the HESCs (*see* **Note 9**).

1. Trypsinize mouse ES cells and plate individual cells in pregelatinized 6-cm Petri culture dishes at a low density (1,000 cells per plate).

2. Culture either with the medium that was in current use or the new tested medium at 37 °C in a 5 % CO_2 atmosphere (*see* **Note 10**).

3. Change medium once on the fifth day after plating.

4. On the seventh day, rinse the cultures with PBS and stain for 5 min with 0.15 % Leishman's fix and stain.

5. Wash the stained cultures thoroughly with water and let them air-dry.

6. Compare the number of colonies per plate as well as the size and degree of differentiation and select the batch of serum with the best performance compared with the batch in use.

3.2 Transfection (See Table 1 and Fig. 1)

3.2.1 DNA Preparation for Transfection

1. Prepare DNA vector by any commonly used technique to obtain OD280/OD260 absorption ratio value of 1.8 or greater (*see* **Note 11**).

2. To linearize the vector by digesting it with the appropriate restriction enzyme.

3. Assess the completion of the restriction digest by electrophoresis of a small aliquot on a 1 % gel agarose.

4. Ethanol precipitates the DNA and resuspend in a small volume (20–50 μL) of TE or sterile water. Adjust concentration to 1 μg/μL.

Table 1
Transfection protocol timetable

Days	
1	Plate MEF-resistant cells
2	Split/thaw a vial of HESC to high density
4	Transfect HESCs (high density cultures of 8–32 cells/colony)
5	Begin selection
6–10	Change selection media every day
11–15	Change selection media every other day
16–18	Screen for resistant colonies Pick up selected colonies and plate them on MEF-resistant feeder in 1× 24-well tissue culture trays
20–30	Split 1:2 and plate on MEF-resistant feeder in 1× 12-well twice Freeze and/or screen/further propagate in 1× 6-well trays

MEF mouse embryonic fibroblasts, *HESC* human embryonic stem cell

Fig. 1 Schematic illustration describing the methods for generating genetically modified HESCs by transfection

3.2.2 Preparing HESCs for Transfection

1. Grow healthy and undifferentiated cells and split (1:2 or 1:3) 2 days before transfection with Collagenase (*see* **Note 12**).

2. Collect HESC culture (70–80 % confluence) by Collagenase treatment into a 15 mL Falcon tube (*see* **Note 7**).

3. Let cell clumps sink to the bottom of the tube for 10–15 min.

4. Aspirate supernatant and gently rinse with PBS.

5. Centrifuge cells at 600 × g for 5 min and aspirate supernatant to obtain a cell pellet.

Transfection with Mirus (TransIT-LT1) Transfection Reagent (*See* Note 14)

1. Warm TransIT-LT1 reagent to room temperature and vortex gently before use.

2. For each well of a six-well tissue culture tray prepare a sterile tube containing 250 μL of Opti-MEM I.

3. Add 2.5 μL of DNA (1 μg/μL stock). Pipette gently to mix.

4. Add 7.5 μL TransIT-LT1 reagent to the diluted DNA mixture. Pipette gently to mix.

5. Incubate TransIT-LT1:DNA complex at room temperature for 15–30 min.

6. Add TransIT-LT1:DNA complex on cell pellet.

7. Resuspend cells with the transfection complex with fresh growth media without Pen-Strep (*see* **Notes 13–15**).

8. Plate on drug-resistant MEFs following a 1:3 split, and incubate for 24–48 h.

9. Change to fresh media with Pen-Strep and appropriate selection drug.

10. Change drug containing HESC media once a day (5 days) and then every other day for a period of approximately 10 days, until resistant HESCs colonies begin to appear.

Electroporation (Essentially According to Zwaka and Thomson)

1. Grow healthy and undifferentiated cells in a 6-well tray until they reach cell density greater than 70 % confluence (*see* **Note 12**).

2. Trypsinize cells to collect clumps of undifferentiated HESC by adding 0.5 mL per well of TrypLE for 5 min (*see* **Note 16**).

3. Add 1 mL HESC growth medium to each well.

4. Collect cell suspension into a 15 mL Falcon tube.

5. Centrifuge cells at 600 × g for 5 min.

6. Aspirate supernatant and gently resuspend in 0.8 mL of HESC fresh media, containing 20–30 μg linearized DNA vector, to reach a final cell concentration of $1–3 × 10^7/0.8$ mL.

7. Transfer cell/DNA mix into precooled 0.4 cm cuvettes.

8. Electroporate cells using the following parameters: 320 V, 250 μF. The time constant should be between 9.0 and 13.0 (*see* **Note 17**).

9. Immediately after electroporation, allow cells to recover by standing in the cuvette on ice for 10 min

10. Transfer contents, using 1 mL glass pipette, into 15 mL tube containing 2 mL of prewarmed HESC media.

11. Pellet cells by centrifugation of $600 \times g$ for 5 min.

12. Aspirate supernatant and gently resuspend pellet in 10 mL HESC media in the presence of 1 μL/1 mL of ROCK inhibitor (10 μM stock).

13. Plate cells on to two 10 cm culture dishes pre-seeded with 2.5×10^6 inactivated MEF feeders and return to incubator.

14. The following day remove cell debris by washing twice with PBS and then add fresh HESC media.

15. Apply selection the following day (day 2 post electroporation).

16. Change drug containing HESC media once a day (5 days) and then every other day.

3.3   Infection
(See Table 2 and Fig. 2)

*3.3.1   Retrovirus/
Lentivirus Production*

1. Plate 293T cells in 10 mm tissue culture dish (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FBS, glutamine, Pen-Strep) 24 h before transfection so that they are 80 % confluent for transfection.

2. Cotransfected 293T cells with 3 μg retroviral/lentiviral vector, 2 μg packaging plasmid, 1 μg VSV-G expression vector and 18 μL TransIT-LT1 (Mirus) per plate according to the suppliers conditions. Transfection of the cells has to be done in medium without antibiotics.

3. After 24 h change medium to full medium (with antibiotics).

Table 2
Infection protocol timetable

Days	
1	Plate 293T cells 2×10^6 cells per plate
2	Transfect the 293T cells with the viral vectors (TransIT-LT1) Split/thaw a vial of HESC to high density
3 4	Change the medium of the 293T cells Filter the viral supernatant (48 h) and infect the HESCs Add new medium to the 293T cells
5	Filter the viral supernatant (72 h) and infect the HESCs
6–10	Change selection media every day
11–15	Change selection media every other day
16–18	Screen for resistant colonies Pick up selected colonies and plate them on MEF-resistant feeder in $1\times$ 24-well tissue culture trays
20–30	Split 1:2 and plate on MEF-resistant feeder in $1\times$ 12-well twice Freeze and/or screen/further propagate in $1\times$ 6-well trays

MEF mouse embryonic fibroblasts, *HESC* human embryonic stem cell

Fig. 2 Schematic illustration describing the methods for generating genetically modified HESCs by infection

4. Collect virus supernatant from all plates 48 h and 72 h after transfection with plastic pipettes and filter supernatant through a 0.45 µm filter.

3.3.2 Retroviral and Lentiviral Gene Transfer into Human ES Cells

1. Cultivate HESCs cultures on mouse embryo fibroblast feeder cells (MEF) or on matrigel in basic fibroblast growth factor (bFGF) supplemented MEF conditioned medium.

2. Plate 1×10^5 HESCs on a tissue culture plate pretreated with Matrigel or Gelatin and MEF attached cells. In the case of Matrigel add MEF conditioned medium supplemented with bFGF (4 ng/mL) to keep the HESCs undifferentiated.

3. Collected and filtered the viral supernatant, after 48 h of cells transfection, together with 6 µg/mL Hexadimethrine Bromide (polybrene).

4. Culture the cells with the virus for 24 h, wash three times with PBS, and then add fresh media or the 72 h viral supernatant, for another 24 h in order to increase the infection efficiency.

5. On day 3 after infection, measure for transgene activity and continue the culture on MEFs or matrigel.

3.4 Colony Picking and Expansion

After 10–12 days in selection media, individual HESC-resistant clones become visible and are big enough to be isolated for expansion.

1. Screen transfected culture plates using an inverted microscope for the presence of resistant clones and mark their location at the bottom of the dish.

2. Manually pick selected HESC colonies (*see* **Note 18**).

3. Disconnect the cell colony from the feeders by dissociating it into small cell pieces using the sharp edge of the glass micropipette while collecting them by aspiration into the tip of the pipette.

4. Plate the small cell clumps on fresh drug-resistant feeder layer, in a single well of a 24-well culture tray and return to incubator for further growth. The replated cell clumps, which have originated from a single cell clone, give rise to round flat colonies with well-defined borders in 3–5 days, while changing the selection media as necessary (*see* **Notes 18–20**).

5. Scale up the clone population by splitting 1:2 with trypsin, twice.

6. When the wells (2 × 12-well) are approaching confluence, freeze each well in individual cryovial. The remaining cells can be either further expanded (Fig. 3c), by splitting 1:4 or directly used for DNA, RNA, or protein extraction (*see* **Note 18**) (Table 1).
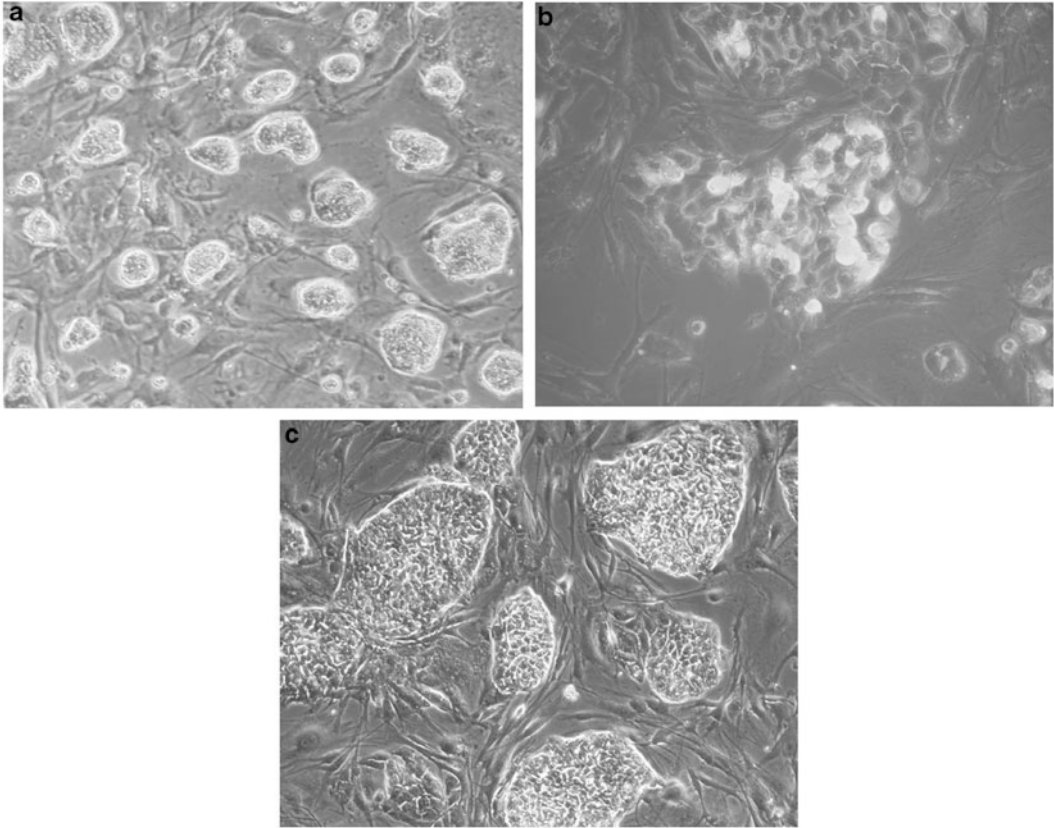
Fig. 3 (**a**) Human embryonic stem (HESC) cell culture on day of transfection. The culture should be composed of many small (8–32 cells) colonies. (**b**) Transient expression of CMV-EGFP in HESCs after 48 h to transfection. (**c**) Established cell line of HESCs after transfection, selection, and clonal expansion of genetically modified cells

4 Notes

1. Section 2.1, **items 1–14** are stored at 4 °C, **items 15–24** at −20 °C, and **item 25** at room temperature. As a rule, all tissue culture protocols must be performed under sterile conditions, in a laminar flow hood, using sterile disposable plastics and clean, detergent-free, glassware.

2. Media should be stored in 4 °C and can be used for up to 1 month.

3. Serum replacement is sensitive to light. Protect supplemented HESC media by covering it with aluminum foil.

4. The mouth-controlled device is the same as the one that is commonly used for handling oocytes and preimplantation embryos in mice. The mouthpiece is available as a part of an aspiration tube assembly from Drummond (model no. 2-000-0001). Sterile glass Pasteur pipettes are pulled on a flame to

create long tubing with a narrow opening. Soften the glass tubing by rotating it in a fine flame until the glass becomes soft. Then, withdraw the glass quickly from the heat and pull both ends smoothly to produce a tube with an internal diameter of about 200 μm. Neatly break the tube and fire polish its tip by quickly touching the flame.

5. All tissue culture procedures are performed under sterile conditions, using prewarmed media and gelatin-precoated plates.

6. As in other cell lines growing in vitro, chromosomal aberrations may occur. Working with cells of low passage number can minimize this. Thus, it is advisable to monitor the karyotype of the cells following prolonged growth in culture and subsequent to stable transfection.

7. It is essential that the HESCs remain as small cell clumps (5–10 cells). Avoid dissociation of the HESCs to single cells when splitting.

8. ROCK Inhibitor Y27632 is a selective inhibitor of the Rho-associated kinase p160ROCK. Treatment with ROCK Inhibitor Y27632 prevents dissociation-induced apoptosis of human embryonic stem cells (HESCs), increasing the survival rate and maintaining pluripotency during freezing and thawing of HESCs.

9. Cell thawing must be performed as quickly as possible.

10. The culture medium is supplemented with 10 % of the tested batch of knockout serum substitute (instead of 15 %) and mouse recombinant LIF at 1,000 U/mL.

11. The purity of the DNA is very critical for successful transfection.

12. The cells should be transfected during the lag phase of cell division. The transfection rate is most efficient when the cell density reaches 50–70 % and the colonies are small (8–32 cells per colony) (Fig. 3a). The colonies should have discrete borders and be composed of similar sized cells, with a pronounced nucleus.

13. Antibiotics will inhibit transfection complex formation and therefore should be excluded from the HESC growth media until the following day.

14. Alternatively, transfection complexes can be added directly to the cells as they grow in culture. However, this may reduce transfection efficiency.

15. In parallel to the experiment, one may consider to carrying out transient transfection on a small number of cells with a construct carrying a constitutive expressed reporter gene, such as CMV-EGFP, to assess transfection efficiency before applying selection (Fig. 3b).

16. For electroporations, it is necessary to dissociate cells to single cells suspension. Therefore, it is essential to trypsinize the cells with TrypLE and then resuspend them with media supplemented with ROCK inhibitor (1 µL/1 mL of ROCK inhibitor from a 10 µM stock) to prevent from cell death associated with colony dissociation.

17. There are various apparatuses that can be applied for electroporation in HESCs. Therefore, electroporation parameters may change and must be adjusted accordingly.

18. The colonies are picked up by the aid of a mouth apparatus connected to a sterile pulled and fire polished Pasteur pipet, as is commonly used for handling oocytes and preimplantation embryos (*see* **Note 4**).

19. We find this pickup method more suitable and efficient for isolating single HESC colonies than the method applied in mouse, where individual ES colonies are collected with a disposable tip, trypsinized, and then plated.

20. In some cases, it is crucial that no feeders will be present during the screen. For this purpose, cells must be propagated in feeder-free culture conditions, for at least one passage. Under such conditions the cells must be grown on vitronectin or matrigel-coated plates, preventing from differentiation and consequently culture loss.

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Genetic Modification in Human Pluripotent Stem Cells by Homologous Recombination and CRISPR/Cas9 System

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Abstract

Genetic modification is an indispensable tool to study gene function in normal development and disease. The recent breakthrough of creating human induced pluripotent stem cells (iPSCs) by defined factors (Takahashi et al., Cell 131:861–872, 2007) provides a renewable source of patient autologous cells that not only retain identical genetic information but also give rise to many cell types of the body including neurons and glia. Meanwhile, the rapid advancement of genome modification tools such as gene targeting by homologous recombination (Capecchi, Nat Rev Genet 6:507–512, 2005) and genome editing tools such as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR-associated) system, TALENs (Transcription activator-like effector nucleases), and ZFNs (Zinc finger nucleases) (Wang et al., Cell 153:910–918, 2013; Mali et al., Science 339:823–826, 2013; Hwang et al., Nat Biotechnol 31:227–229, 2013; Friedland et al., Nat Methods 10(8):741–743, 2013; DiCarlo et al., Nucleic Acids Res 41:4336–4343, 2013; Cong et al., Science 339:819–823, 2013) has greatly accelerated the development of human genome manipulation at the molecular level. This chapter describes the protocols for making neural lineage reporter lines using homologous recombination and the CRISPR/Cas system-mediated genome editing, including construction of targeting vectors, guide RNAs, transfection into hPSCs, and selection and verification of successfully targeted clones. This method can be applied to various needs of hPSC genetic engineering at high efficiency and high reliability.

Keywords: Gene targeting, Reporter cell line, Genetic engineering, Human induced pluripotent stem cells, Genome editing, Homologous recombination, ZFN, CRISPR, Cas, TALEN

1 Introduction

Genetic modification is an indispensable tool to study gene function in normal development and disease. The recent breakthrough of creating human induced pluripotent stem cells by defined factors (1) and human embryonic stem cells (collectively called hPSCs) provides a renewable source of patient autologous cells that not only retain identical genetic information but also give rise to many cell types of the body including neurons and glia, two major cell types in the central nervous system (CNS). Meanwhile, the rapid advancement of genome modification tools such as gene targeting by homologous recombination (2) and genome editing tools including CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR-associated) system, TALENs

(Transcription activator-like effector nucleases), and ZFNs (Zinc finger nucleases) (3–8) has greatly accelerated the development of human genome manipulation at the molecular level. This chapter describes the protocols of making neural lineage reporter lines using homologous recombination and CRISPR/Cas system-mediated genome editing, including construction of targeting vectors and guide RNA vectors, transfection into hPSCs, and selection and verification of successfully targeted clones. The reporter proteins (fluorescent proteins, drug-resistant cassettes or other tags) are driven by the endogenous promoters and therefore recapitulate the endogenous gene expression both spatially and temporally. This method can be applied to various needs of hPSC genetic engineering at high efficiency and high reliability. This protocol has been successfully implemented in targeting multiple hPSC lines including iPSC lines derived from Down syndrome patients, amyotrophic lateral sclerosis (ALS) patients, and healthy individuals, as well as human embryonic stem cell (hESC) lines BG01, BG01V, and WA09 (H9), for a variety of purposes. Using this protocol, we have created multiple neural lineage-specific hPSC reporters including HB9-GFP, OLIG2-GFP, NEUROG2-mCherry-hygromycin, and SOX1-GFP-neomycin knockin lines, and have performed gene correction experiments for SOD1 point mutations in ALS patient iPSCs. In addition, using the same strategy, we have generated safe harbor targeting and floxing in hPSCs, a system that allows for subsequent recombinase-mediated cassette exchange (RMCE) and other complicated genetic modifications in hPSCs.

2   Materials

2.1   Targeting Vector Components

2.1.1   Vector Collection

1. pStart-K (Addgene, cat. no. 20346).
2. pWS-TK6 (Addgene, cat. no. 20350).
3. pKD3 (Addgene, cat. no. 45604).
4. pKD46 (The Coli Genetic Stock Center, CGSC cat. no. 7634).
5. EGFP cassette.
6. IRES-mCherry-IRES-Hygromycin cassette.
7. PGK-neo-bpA sequence (Addgene, cat. no. 13442).
8. Human BAC clones of target genes (https://bacpac.chori.org).

2.1.2   CRISPR/Cas9 System Vector Collection

1. JDS246 (Cas9-003), Mammalian codon-optimized *streptococcus pyogenes* Cas9-3X Flag (Addgene, cat. no. 43861).
2. MLM3636, Human-gRNA-Expression Vector with U6 promoter (Addgene, cat. no. 43860).
3. gRNA design: http://zifit.partners.org/ZiFiT/

4. Off-target prediction tool: http://eendb.zfgenetics.org/casot/download.php

5. gRNA Primer synthesis: Sigma.

2.1.3 Molecular Biology Reagents

1. Competent cells: One shot Top10 Electrocomp *E. coli* (Life Technologies, cat. no. C4040-50).

2. AccuPrime Pfx SupperMix (Life Technologies, cat. no. 12344–040).

3. Restriction enzymes (NEB and Life Technologies).

4. Zymoclean Gel DNA Recovery Kit (Zymo Research, cat. no. D4007).

2.2 Cell Culture

1. Stempro hPSC SFM medium (Life Technologies, cat. no. A1000701).

2. Essential 8 medium (Life Technologies, cat. no. A14666SA).

3. Dulbecco's Phosphate Buffered Saline without Calcium and Magnesium (Life Technologies, cat. no. A12856-01).

4. D-MEM/F12 with Glutamax (Life Technologies, cat. no. 10565018).

5. D-MEM with Glutamax (Life Technologies, cat. no. 10566040).

6. Fetal Bovine Serum-ES cell qualified (Life Technologies, cat. no. 10439).

7. Knockout serum replacement (Life Technologies, cat. no. 10828010).

8. 2-Mercaptoethanol 1,000× (Life Technologies, cat. no. 21985023).

9. Non-Essential Amino Acid (Life Technologies, cat. no. 11140050).

10. Stempro Accutase (Life Technologies, cat. no. A1110501).

11. Dispase (Life Technologies, cat. no. 17105–041).

12. 0.25 % Trypsin–EDTA solution (Life Technologies, cat. no. 25200–056).

13. Geltrex (Life Technologies cat. no. 12760–013).

14. ROCK inhibitor (Y-27632, Millipore, cat. no. SCM075).

15. SMC4 reagent (BD, cat. no. 354357).

16. Neomycin-resistant MEF (Millipore, cat. no. PMEF-NL).

17. Hygromycin-resistant MEF (Millipore, cat. no. PMEF-HL).

18. Neomycin-, hygromycin-, and puromycin-resistant MEF can be made from DR-4 mouse strain as multiple drug-resistant MEFs (Jackson laboratory, Tg DR4 1Jae/J, Stock Number: 003208).

19. G418 (Geneticin, Life Technologies, cat. no. 11811).

20. Hygromycin B (Life Technologies, cat. no. 10687010).

21. FIAU (Fialuridine, 1-(2-Deoxy-2-fluoro-ß-D-arabinofurano-syl)-5-iodouracil, Moravek Biochemicals and Radiochemicals, cat. no. M251).

22. *hPSC cell lines*: ND2.0, ND1.4, house reprogrammed iPSCs.

2.3 Electroporation Equipment and Reagents

1. Electroporator: Gene Pulser Xcell (Bio-Rad).

2. 0.4 cm electroporation cuvette (Bio-Rad, cat. no. 165–2088).

2.4 Genomic PCR and Southern Blot

1. DIG-High prime DNA labeling and detection starter kit II (Roche, cat. no. 11585614910).

2. Hybridization denature solution (VWR, cat. no. 82021–478).

3. PCR DIG probe synthesis kit (Roche, cat. no. 11636090910).

4. DIG wash set (Roche, cat. no. 11585762001).

5. Anti-Digoxigenin (DIG)-AP (Roche, cat. no. 11093274910).

6. CSPD chemiluminescence system (Roche, cat. no. 11755633001)

7. DIG wash and block buffer set (Roche, cat. no. 11585762001).

8. 50× TAE buffer (Life Technologies, cat. no. 24710030)
   Blotting buffer (25 mM Tris pH 7.4, 0.15 M NaCl, 0.1 % Tween20).

9. Hoefer Ultraviolet Crosslinker (Fisher Scientific, cat. no. 03-500-308).

10. Spermidine (Fisher, cat. no. AC13274-0010).

11. Tris–HCl 2 M, pH 7.5 (VWR, cat. no. 200064–506).

12. Denville Scientific blue bio film 8 × 10 (Fisher, cat. no. nc9550782).

13. DNA molecular weight marker II, DIG-labeled (VWR, cat. no. 11218590910).

14. Amersham Blotting membrane Hybond-N+ (Roche, cat. no. 95038–400).

15. Pyrex glass drying tray (Fisher, cat. no. 15-242A).

16. Kimberly-Clark C-fold paper towels (Fisher, cat. no. 06-666-32B).

17. Whatman 3MM paper, 26 × 41 (Fisher, cat. no. 05-713-336).

18. Hybridization bag (Roche, cat. no. 11666649001).

19. Hybridization tubes (Fisher, cat. no. 13-247-300).

20. Hybridization oven rotisserie Shake "n" Stack (Fisher, cat. no. HBMSOV14110).

3 Methods

3.1 Vector Construction

3.1.1 General Suggestions

Vector construction protocol follows the published instructions (9–11). Briefly, the targeting vectors are designed to have a floxed positive selection (usually is an antibiotic resistant) cassette and a negative selection (thymidine kinase, TK, or diphtheria toxin A, DTA (12)) cassette, which is located right outside of the 3′ homology arm. To enhance negative selection, an additional copy of DTA can be added upstream of the 5′ homology arm. Reporter genes include a variety of gene cassettes, with different flavors of fluorescent proteins (e.g., green or red fluorescent proteins), lacz, drug selection, etc. The purpose of using gene targeting is to target the reporters or dual reporters precisely to the endogenous locus. To prevent the interference of fusion reporter proteins with the expression of gene of interest (GOI) and to preserve both copies of GOI and avoid haploinsufficiency, we choose to construct targeting vectors with GOI tagged by a fluorescent protein and a drug selection, which are connected to the GOI sequentially by an IRES (internal ribosome entry site) cassette. Alternatively, the self-processing viral 2A peptides, F2A (from the foot-and-mouth disease virus), E2A (from the equine rhinitis A virus), T2A (from the *Thosea asigna* virus), or P2A (from the porcine teschovirus) (13, 14) can be used in the place of IRES. These strategies allow for the concurrent expression of fluorescent proteins and drug selections under the promoter of the endogenous GOIs. An example is shown in Fig. 1 for constructing targeting vector for human SOX1 gene, tagged by EGFP and neomycin resistance cassette.

Briefly, a human BAC clone containing the GOI is purchased from Life Technologies or Children's Hospital and Research Center at Oakland (https://bacpac.chori.org). The clone is verified by PCR amplification of the gene. The targeting vector is constructed in DH5α using recombineering and multisite gateway as described (9, 10). The resultant constructs have the following format: 5′ homo arm-endogenous GOI-IRES-Fluor protein-IRES-Drug selection-3′ homo arm-TK. After linearization, targeting vectors are electroporated into hPSCs.

3.1.2 Design and Vector Construction for Cas9 and gRNA for GOI

Recently we have quickly implemented the CRISPR/Cas (3–8) system in the lab, by which one-step concurrent mutation for multiple genes has been reported in the mouse (3). We have designed and obtained multiple guide RNAs (gRNAs) (Table 1 shows an example of gRNAs designed for genetic correction of SOD1 protein at the 139th amino acid of N→K) using Golden Gate (15–21) cloning. To precisely target to GOI, we have applied the following rules in CRISPR designs and practice. (a) Improve specificity and minimize off-target mutagenesis by choosing unique "seed sequence" with low GC and high AT content, and use the
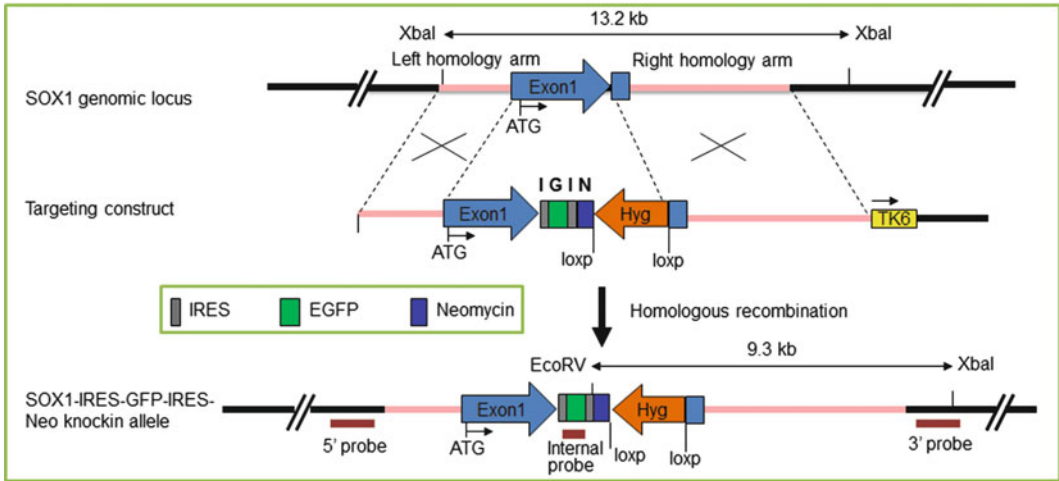
Fig. 1 Targeting vector design for creating human SOX1 gene knockin reporter hPSC line. SOX1 genomic sequence is tagged by dual reporter cassettes, EGFP and neomycin resistance, both of which are connected by IRES and therefore driven by the endogenous SOX1 promoter. EGFP and neomycin can both be used for selection and purification of SOX1 expressing cells. Please note that Hygromycin resistance and TK6 cassettes are included for positive and negative selections, respectively, during isolation of targeted hPSC clones

Table 1
Guide RNA (gRNA) sequence for genetic correction of SOD1 N139K mutation

Name	Sequence 5′→3′
SOD1_N139K_gRNA1	GCTGGAAGTCGTTTGGCTTGTGG
SOD1_N139K_gRNA2	GCAGATGACTTGGGCAAAGGTGG
SOD1_N139K_gRNA3	GGGCCTCAGACTACATCCAAGGG

minimal combined amount of gRNA and Cas9 plasmids. (b) Optimize the ratio of hiPSC number and the amount of total plasmid DNA of gRNA and Cas9. Based on the literature (3) and our own experience, for 1×10^6 hiPSCs, we will use 5 μg gRNA expression vector and 5 μg Cas9 expression vector total. (c) Rank gRNAs for each gene based on indel % from SURVEYOR assay (Fig. 2), and choose the gRNAs with "medium" efficiencies to avoid off-target events. (d) Monitor the number of targeted copies by Southern blot. Potential off-target sites can be predicted using an online open source tool http://eendb.zfgenetics.org/casot/download.php, which is a Perl-based program (http://www.perl.org/get.html). An example of potential off-target sites for the gRNAs designed for genetic correction of SOD1 mutation (N139K) is listed in Table 2.
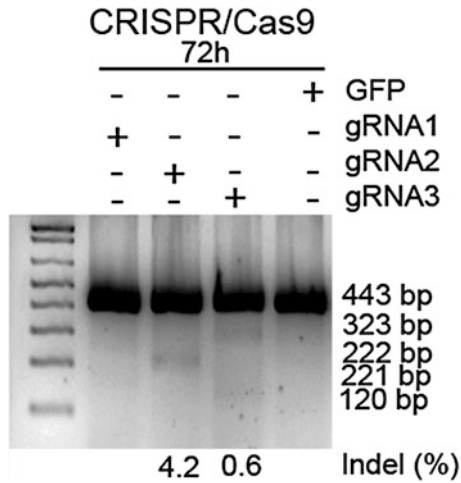
Fig. 2 SURVEYOR assay of guide RNA (gRNA)-mediated cleavage in 293 cells by the CRISPR/Cas9 system. The gRNAs are designed to facilitate gene correction of a point mutation of AAC→AAA that changes the 139th aa from N(Asn) to K (Lys) in the human SOD1 gene in Amyotrophic Lateral Sclerosis (ALS) patient iPSCs. gRNA2 and gRNA3, which result an indel of ~4 % and 0.6 % respectively, can be used for gene correction in ALS SOD1 N139K patient iPSCs

3.2 Transfection of hPSC to Create Homologous Recombinants

3.2.1 Culture hPSCs on MEF

Single cells are needed for electroporation to generate homologous recombinants. hPSCs cultured on mouse embryonic fibroblasts (MEFs) can be adapted to feeder-free MEF-conditioned medium, and passaged in a single cell fashion for electroporations. Commercially available, chemically defined media such as Stempro SFM (Life Technologies) and mTesR (Stem Cell Technologies) can also be used for subculture. Usually it takes 2–3 days for the cells to get fully adapted to a new type of medium; therefore, constantly changing culture medium type is not recommended. After electroporation, cell recovery is improved if they are plated onto MEF in MEF-conditioned medium with ROCK inhibitor or SMC-4 (Fig. 3). Therefore maintaining the cells on MEF or in MEF-CM is optimal for this particular set of electroporation experiments. If chemically defined medium other than MEF-CM is used, give the cells an additional 2–3 days for adaptation. Essential 8 medium (Life Technologies), another very popular hPSC medium, does not support single cell passage; therefore, it can only be used for intermediate steps of subculture.

1. MEFs are prepared from E13.5 mouse embryos and cells from passage 1 saved in the liquid nitrogen storage. Cells of up to passage 3 can be inactivated by irradiation which can then be used as feeders to support hPSC growth. The irradiation dosage is 8000 Rad (cGy). Inactivated MEFs can be aliquoted as desired and stored in liquid nitrogen.

2. The day prior to thawing the hPSCs, seed inactivated MEF dishes at a density of 3×10^4 cells/cm^2 in MEF medium.

Table 2
Potential off-target sites for guide RNAs (gRNA2 and gRNA3) designed for gene correction of SOD1 N139K by gene targeting

gRNA	Coordinate (chromosome: location:strand)	Sequence	Mm (seed)	Mm (nonseed)	Exon information
gRNA2	8:126964170–126964193:−	cCAGATGA_CTTGGG CAAAGa-TGGA	1	1	SOD1P3
gRNA2	14:69520998–69521021:+	cCAGATGc_CTTGGGgAc AGG-AGGA	2	2	DCAF5
gRNA2	15:62360764–62360787:−	GCAGAaGt_CcTGGtCA AAGG-AGGC	2	2	C2CD4A
gRNA2	16:25157949–25157972:−	GCtGtTGA_CTTtGGC AgAGG-CGGC	2	2	RP11-266L9.2
gRNA2	19:53594799–53594822:+	GCgGggGA_CTTGGGg AAAGG-GGGC	1	3	ZNF160
gRNA2	2:132238074–132238097:−	tgAGATGA_CTgGGGC AtAGG-TGGC	2	2	TUBA3D
gRNA2	7:27788177–27788200:−	aaAGATGA_CaTGGG CAAAGt-TGGA	2	2	TAX1BP1
gRNA2	7:96653811–96653834:+	GCtGAaGA_CTcGGG CAAAGt-TGGC	2	2	DLX5
gRNA3	16:19098321–19098344:+	aGGCaTCA_GACaA CATCCAA-GGGG	1	2	RP11-626G11.4

Notes. Mm mismatch
Generally, if the combined number of Mms within seed and nonseed sequence is >4, off-target activities are unlikely. If the number of Mms within the seed sequence is <2, off-target events need to be monitored and examined
Mismatched bases are in lower case

3. Take hPSCs (~2 × 10^6 cells/vial) from liquid nitrogen storage to 37 °C water bath immediately. Transfer all contents from the vial to a 50 mL conical tube and add prewarmed hPSC growth medium dropwise up to 10 mL.

4. Spin at 200 × g for 4 min at room temperature and aspirate the supernatant.

5. Resuspend cells in 1 mL of hPSC growth medium and plate onto the MEF dish prepared earlier (step 2).

6. When cells are ready to be passaged, aspirate medium and add 3 mL dispase solution per 100 mm dish.

7. Incubate until the edges of colonies start to curl up.

8. Gently triturate the clumps using a 5 mL serological pipette and transfer the clumps onto a 15 mL conical tube.
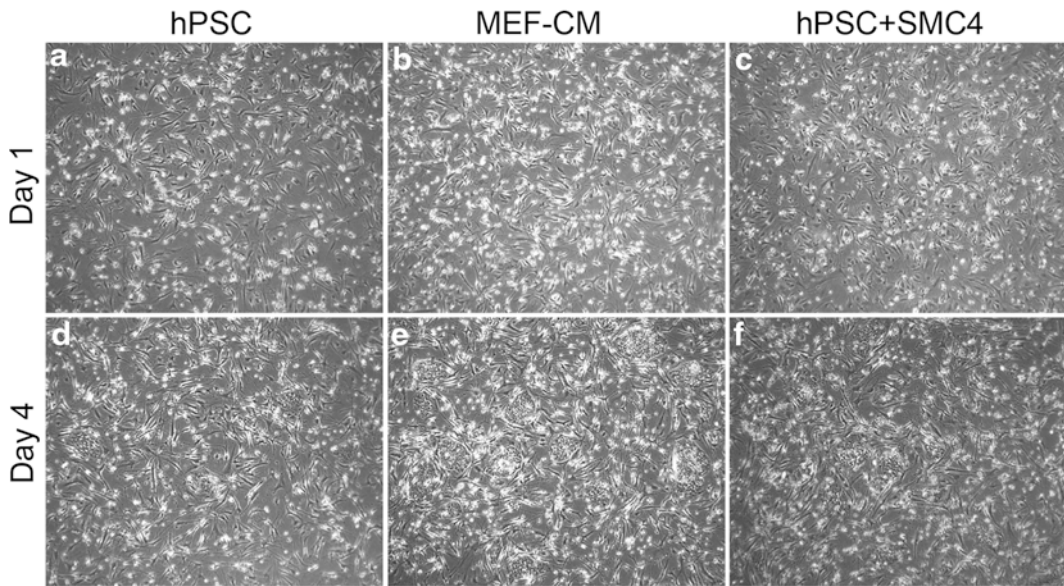
Fig. 3 SMC4 improves single cell survival after electroporation. Equal number of hPSCs were plated after electroporation in hPSC medium (**a**, **d**), or MEF-conditioned hPSC medium (MEF-CM, **b**, **e**), or hPSC medium supplemented with SMC4 reagent (SMC4, **c**, **f**). Significantly higher number of cells and colonies appeared in MEF-CM and SMC4 groups at both day 1 and 4 postelectroporation

9. Spin at $200 \times g$ for 4 min at room temperature and aspirate the supernatant.

10. Gently aspirate medium and resuspend the cells in hPSC growth medium.

11. Transfer the cell clumps onto new MEF dishes at a ratio of 1:4, every 4–5 days.

3.2.2 Electroporation

Transfer of hPSCs onto Geltrex-Coated Dishes for Feeder-Free Culture

1. Preparation of Geltrex-coated dishes. Thaw a whole bottle of Geltrex at 4 °C for 3 h or overnight to prevent polymerization (*see* **Note 1**).

2. Add equal volume of cold DMEM/F12 to make $100\times$ stock solution and store desired aliquots at -20°C.

3. Before coating plates, thaw aliquots at 4 °C or on ice. Add appropriate volume of DMEM/F12 to make $1\times$ solution and add to culture dishes to completely cover the surface area (e.g., 2–3 mL/60 mm dish), and incubate at room temperature for 1 h.

4. Coated dishes can be used immediately after coating or stored at 4 °C for 2 weeks.

5. Avoid drying the dishes and remove coating solution immediately before use.

6. Passage of hPSCs onto Geltrex-coated dishes: Harvest cells following Section 3.2.1, steps 6–11.

7. Instead of spinning down the cell pellets, let the cells settle to the bottom of the tube by allowing the tube to sit in the tissue culture hood for 5–10 min (*see* **Note 2**).

8. Remove supernatant, and aliquot cells at 1:4 ratio onto Geltrex-coated dishes with MEF-CM supplemented with fresh bFGF (12 ng/mL).

9. When cells reach ~80 % confluency, seed the hPSC onto five 10-cm, Geltrex-coated dishes with CM with fresh bFGF (12 ng/mL). When cells reach ~80% confluency, they can be used for electroporation.

3.2.3 Electroporation, Antibiotic Selection, and Isolation of Clones

1. One day before electroporation, prepare six 10-cm *drug*-resistant MEF dishes (*see* **Note 3**).

2. Electroporation. Prewarm 12 mL of MEF-CM in a 15 mL tube at 37 °C.

3. Aspirate medium from Geltrex-coated plate, add 2.5 mL accutase per 10 cm dish (*see* **Note 4**) before adding to dishes. Leave it at room temperature for 3–5 min until the cells dislodge.

4. Collect cells by adding 5 mL DMEM/F12 medium, wash once, and collect everything into a 15 mL conical tube. Take an aliquot of cells for counting.

5. Aliquot 1×10^7 cells to a 15 mL conical tube containing 10 mL DPBS, centrifuge at $200 \times g$ for 5 min. Remove supernatant as much as possible. Resuspend cells with 700 µL DPBS (the total final volume is 800 µL).

6. Add 50 µg linearized targeting vectors and mix well. Transfer the cell mixture to a 0.4 cm cuvette.

7. Alternatively, if CRISPR/Cas9 system is used, prepare 1×10^6 cells, and electroporate 5 µg Cas9 vector, 5 µg gRNA vector, and 5 µg targeting (donor) vector. Proceed to step 8 (*see* **Note 5**).

8. Electroporate cells using the following parameters: 250 V, 250 µF, $\infty \Omega$. Immediately transfer the cells to a 15 mL conical tube containing 12 mL MEF-CM. Mix the cells, put it at room temperature for 5 min and mix the cells gently once per minute by tapping the tube during incubation.

9. Transfer the cells to six 10-cm *drug*-resistant MEF dishes containing 8 mL MEF-CM. Add FGF so that the final FGF concentration is 12 ng/mL. Return the cells to the incubator at 37 °C with 5 % CO_2.

10. To enhance single cell survival, 10 μm ROCK inhibitor or SMC-4 can be added for the first 24 h post electroporation (*see* **Note 6**).

11. Change medium everyday for day 1–3 with 10 mL MEF-CM containing 12 ng/mL bFGF.

12. From day 4 to 8, change medium everyday with 10 mL freshly made hPSC medium containing 12 ng/mL bFGF.

13. Individual colonies start to appear around day 3–5. Usually about 200 colonies can be seen by day 7 per 100 mm dish. At this point, start positive selection.

14. Seven days later, *start negative selection* using FIAU (200 nM) together with positive selection in the hPSC medium. Negative selection may last for 2–3 weeks.

15. Culture the cells under both positive and negative selections for 21 days.

16. When individual colonies are visible to the naked eye, but are not merging with neighboring colonies, they are ready to be isolated. Manually isolate single clones using a glass tool made from a Pasteur pipette (*see* **Note 7**) or a 25 G × 1/2 in. syringe needle. Carefully grid and dislodge the colony using the tool, and use a p200 pipette with a 200 μL tip to transfer the dissected clumps into one well of a 24 well plate. Make sure to completely remove all dislodged pieces before dissecting the next clone to avoid cross clonal contamination (*see* **Note 8**).

17. Continue to culture and expand individual clones under positive selection in hPSC medium. Save cell pellets ($2–5 \times 10^5$ cells) to extract genomic DNA for Southern blot analysis to identify homologous recombinants (continue to Section 3.3).

18. For positive clones, split cells every week at a 1:2 ratio. When cells reach to ~5×10^6 (~two 60 mm dishes), freeze and stock 2–3 vials per clone and continue culturing. This takes 2–3 weeks (*see* **Note 9**)

3.3 Identification of Homologous Recombinants

Clones obtained by positive and negative selection will be verified by Southern blot analysis using a nonradioactive protocol with Digoxigenin (DIG)-labeled probe and detection system.

3.3.1 Probe Design

Prepare probes using the DIG-labeling and detection system kit from Roche. Genomic DNA is used as a template to first amplify a nonlabeled probe sequence. 5′-, 3′- and internal probes are designed based on the GOI and the targeting homology arms. The PCR product for probes is then cloned into a TA vector and will be used as a template for making DIG-labeled probes. PCR cycling condition is 95 °C 2 min, 30 cycles of 95 °C 30s → 60 °C 30s → 72 °C 40 s, with final extension at 72 °C for 7 min.

Table 3
Design of Southern blot probes for SOX1-IRES-EGFP-IRES-NEO gene targeting

Probes	Size of probe (bp)	Restriction enzymes	WT band (kb)	Targeted band (kb)
5′	804	EcoRV + XhoI	7.7	9.3
3′	530	EcoRV + XbaI	13.2	9.3
Internal	632	EcoRV + XhoI	7.7	9.3

Notes: Primers (5′→3′) for making 5′ probe: GGTGAGCCCCTACTCCAAAGCT and ATGAGGGGCAAAGAGG-CAGC; 3′ probe: GAAATTACAAAACCAGCTTCCCAGG and TGTACCTCCTCTGCAAAACCTTCCT; internal probe: ATGGTGAGCAAGGGCGAGGA and TTGGGGTCTTTGCTCAGGGC

Table 4
Preparation of DIG-labeled probes

Order	PCR reaction tube	GOI DIG-probe	GOI control	tPA DIG-control	tPA control
1	H_2O, PCR grade	33.25 μL	33.25 μL	29.25	29.25
2	10× PCR buffer	5 μL	5 μL	5 μL	5 μL
3	PCR DIG mix	5 μL	–	5 μL	–
4	dNTP	–	5 μL	–	5 μL
5	10× PCR Primer mix[a]	5 μL	5 μL	–	–
5	Control primer	–	–	5 μL	5 μL
6	Enzyme mix	0.75 μL	0.75 μL	0.75 μL	0.75 μL
7	DNA template[b]	1 μL	1 μL	–	–
7	Control template	–	–	5 μL	5 μL

Notes: [a]10× PCR Primer mix is comprised of both forward and reverse primers at a stock concentration of 5 μM, the final concentration of each primer in reaction mix is 1 μM
[b]If genomic DNA is used, then add 1–50 ng of DNA; If plasmid DNA is used, then add 10–100 pg. Adjust volume to 1 μL

Special attention needs to be paid to the final probe sequence. Excessive consecutive thymidine sequence in the template will result in failure of the integration of DIG-dUTP. An example for designing probes for targeting the SOX1 locus is listed in Table 3.

3.3.2 Preparation of the DIG-Labeled Probe

Reaction mix is prepared as shown in Table 4. The ingredients are added to the reaction tubes (kept on ice) in the order indicated in the table. Preparation of reaction mix.

3.3.3 Examination of the Probe

After PCR, 5 μL of DIG-labeled probe is examined by running on a 1 % agarose gel in 1× TAE buffer. Note that DIG-labeled DNA probe migrates slower than nonlabeled DNA. For instance, the probe amplified from the 5-kb control plasmid that contains the cDNA for the human tissue-type plasminogen activator (tPA) is 442 bp, while the DIG-labeled tPA probe migrates to the

Table 5
Restriction enzyme digestion of genomic DNA from hPSC clones

Ingredients	Amount/reaction (μL)
$10\times$ RE buffer	2.5
$100\times$ BSA	0.5
100 mM Spermidine	1
RE (20 U/μL)	2
H_2O	4
Genomic DNA	15

Notes: *RE* Restriction enzyme
Genomic DNA is adjusted to 300 ng/μL; therefore the total amount is 3–5 μg

500–550 bp position. The 530-bp Olig2 probe migrates to about the 600 bp position once it is DIG-labeled.

3.3.4 Genomic DNA Restriction Enzyme Digestion: Details Below

Genomic DNA (a minimum of 3 μg DNA, from approximately 2–5×10^5 cells) is digested using appropriate restriction enzymes as designed. Ingredients are shown in Table 5.

3.3.5 Electrophoresis and Membrane Transfer

1. Make a 0.9 % TAE agarose gel. Load 2 μL DIG-DNA marker. Load the ordinary, nonlabeled 1 kb DNA ladder in an adjacent lane. Load digested DNA and run the gel at 20–25 V overnight (or 100 V for 2 h). Stain the gel by SYBR green and take the gel image with a UV ruler placed on the side. Remove extra gel that is not going to be transferred.

2. Depurinate DNA if the size of the predicted bands is >5 kb. Soak the gel in 0.2 M HCl for 10 min on a horizontal shaking platform or until the blue dye turns yellow color. Rinse gel with sterile ddH$_2$O.

3. Denature DNA by soaking gel in 0.5 M NaOH, 1.5 M NaCl for 15 min twice, with gentle shaking on a horizontal shaking platform. Rinse gel with sterile ddH$_2$O.

4. Neutralize DNA in 0.5 M Tris–HCl pH7.5, 1.5 M NaCl for 15 min, twice, with gentle shaking.

5. Equilibrate DNA in transfer buffer $20\times$ SSC (0.3 M sodium citrate, 3 M NaCl) for 15 min, with gentle shaking.

6. Soak Hybond N+ membrane in H_2O and then in $20\times$ SSC for 5 min.

7. Set up transfer as shown in Fig. 4.

8. Crosslink the membrane. Place the membrane with the DNA side facing up on top of a wet 3MM paper (previously soaked in ddH$_2$O). Place the paper and membrane on the top of
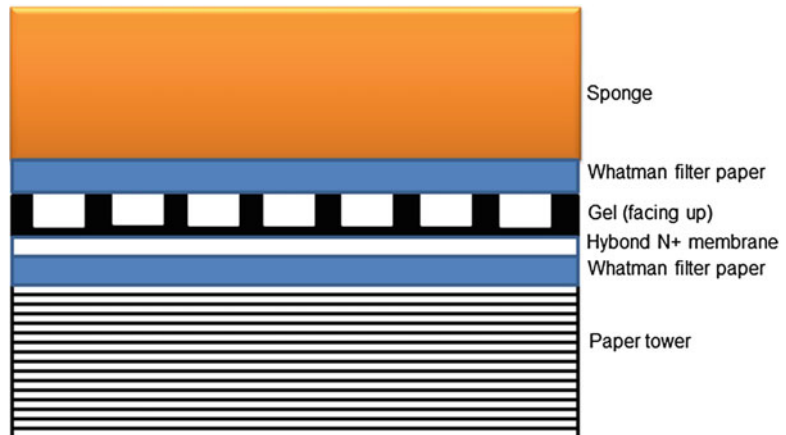
Fig. 4 Scheme for setting up DNA transfer from gel to membrane

XL-1000 UV Crosslinker. Turn on power, press auto crosslink, press start to expose the wet membrane to UV light. Rinse the membrane briefly with ddH$_2$O, air-dry the membrane for 15 min, and store the dry blot between two sheets of 3MM-paper in a sealed bag at 4 °C. The membrane can be stored for up to 1 month.

3.3.6 Prehybridization and Hybridization

1. *Prehybridize using DIG Easy Hyb* (*see* **Note 10**). Heat 25 mL Hyb-granule solution to 45 °C in a 50 mL sterile tube. Fill hybridization tube with sterile ddH$_2$O. Place the membrane into hyb tube with the DNA side facing inward. Ensure that the membrane sticks tightly onto the tube wall with no air bubbles. Remove ddH$_2$O, add 15 mL prewarmed Hyb-granule solution. Incubate at 45 °C for 1 h in hybridization oven with rotation.

2. *Denature the DIG-probe*. Add 20 μL PCR DIG-probe to 50 μL H$_2$O in a 1.7 mL microcentrifuge tube. Boil the DIG-probe for 10 min and chill on ice for 3 min (*see* **Note 11** for conditions for reused probes).

3. Add the denatured DIG-probe to 10 mL prewarmed Hyb-granule solution. Mix well by gentle inversion to avoid foaming as bubbles may increase background.

4. *Hybridization*. Remove Hyb-granule solution from the Hyb tube (*see* **Note 12**), and replace with 10 mL DIG-probe/Hyb mixture to the Hyb tube with membrane. Incubate at 45 °C overnight with rotation.

5. *Posthybridization stringency washes*. Remove DIG-probe/Hyb mixture (*see* **Note 13**). Fill the Hyb tube with 250 mL Wash buffer I ($2\times$ SSC, 0.1 % SDS), wash at 25 °C for 15 min. Repeat the wash once.

6. Fill the tube with 250 mL Wash buffer II (0.5× SSC, 0.1 % SDS), wash at 68 °C for 30 min. Repeat the wash once (*see* **Note 14**).

7. *Immunological detection* (*see* **Note 15**). Remove Wash buffer and replace with 100 mL maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH7.5, 0.3 % Tween 20); gently rotate for 5 min at room temperature.

8. Replace with 120 mL freshly made blocking buffer and gently rotate for 60 min at room temperature (*see* **Note 16**).

9. Replace with 30 mL anti-DIG buffer and gently rotate for 30 min at room temperature (*see* **Note 17**).

10. Replace with 100 mL maleic wash buffer; gently rotate for 15 min at room temperature. Repeat once.

11. Replace with 50 mL Detection buffer (0.1 M Tris pH 9.5, 0.1 M NaCl) and gently rotate for 5 min at room temperature.

12. Place the membrane on a clean plastic sheet (*see* **Note 18**) with the DNA side facing up.

13. Apply 1–2 mL CSPD onto the membrane and cover up with the plastic sheet. Avoid air bubbles.

14. Incubate for 5 min at room temperature.

15. Squeeze out the excess liquid, and seal the plastic sheet with a plastic sealing machine. Incubate for 10 min at 37 °C incubator.

16. Expose to X-ray film for 2 h (at room temperature) to overnight (4 °C).

3.3.7 Blot Stripping and Rehybridization

1. Rinse the membrane with water and wash in stripping buffer (0.2 M NaOH with 0.1 % SDS) at 37 °C for 15 min. Repeat once.

2. Rinse the membrane with 2× SSC for 5 min once.

3. Expose the membrane to X-ray film overnight (as described in Section 3.3.6, step 16) to make sure that no residual signal is present.

4. Proceed to prehybridization and hybridization steps as described in Section 3.3.6 (*see* **Note 19**).

4   Notes

1. When handling Geltrex, keep undiluted Geltrex on ice or in 4 °C at all times.

2. When generate hPSC feeder-free culture from MEF culture, extra care is needed to avoid carrying over MEFs. Letting the pellets settle in the tubes instead of spinning down will largely

eliminate MEFs which are single cells and settle slower than hPSC clumps.

3. The type of drug-resistant MEFs is determined by the vector design for positive selection. Commonly used positive selection cassettes are neomycin, hygromycin, puromycin, and zeocin. Single- or triple-resistant MEFs or mouse strains are available commercially (*see* Materials). Wild type MEFs confer resistance to zeocin at <10 μg/mL, while most unmodified hPSCs will die if cultured in 2 μg/mL zeocin for less than 3 days. Therefore, WT MEFs can be used as feeders for zeocin selection.

4. Accutase should be kept cold at 4 °C.

5. Long-term effects of the Cas9 system in genetic engineering have yet to be comprehensively evaluated.

6. ROCK inhibitor or SMC-4 will only be used for one day (day 1) right after electroporation. Prolonged usage of ROCK inhibitor or SMC-4 might increase the possibility of genetic instability and is not cost-effective. Starting from day 2, regular MEF-CM with 12 ng/mL FGF will be used, and starting from day 8, regular hPSC medium with 12 ng/mL FGF will be used.

7. When isolating individual clones, manual picking is necessary. We have been using 9-in. Pasteur pipettes to make a glass knife, which can grid and cut clones. Alternatively, a p20 pipette tip can be used. Avoid cross clonal contamination. There should be about 180–200 colonies maximum per 10 cm dish.

8. Based on the number of clones, prepare 24 well inactivated MEF plates 1–2 days before planned manual isolation. Usually, at least 50 clones should be isolated. For CRISPR/Cas9 mediated homologous recombination, the targeting efficiency is greatly improved. Positive homologous recombinants should appear in as few as 5–10 colonies. However, because off-target events cannot be completely excluded in CRISPR/ Cas9 targeted clones, to guarantee the identification of qualified homologous recombinants, 10–20 colonies should be isolated.

9. It is good to save cells and pellets from 2 to 4 nonhomologous recombinants. These clones can serve as negative controls.

10. Do not dry the membrane at any time. Wear gloves when handling membranes, gels, and reagents.

11. For reused probes, denature at 68 °C for 10 min. Do not boil.

12. Hyb-granule solution can be saved at −20 °C for reuse.

13. DIG-probe/Hyb mixture may be saved at −20 °C for reuse. When reuse, denature the probe at 68 °C for 10 min. Do not boil.

Table 6
Reagent setup for DIG signal detection in Southern blot analysis

Contents	Working buffer	Storage
DIG Hyb granules (power)	Add 32 mL H_2O, stirring at 37 °C for 5 min, and add 32 mL H_2O again	−20°C
10× Blocking solution	For 15 mL 10× blocking buffer, add 15 mL 10× maleic solution and 120 mL H_2O	Freshly made
Anti-DIG solution	For 3 μL Anti-DIG solution, add 30 mL blocking buffer	Stable for 12 h at 4 °C
CSPD Chemiluminescence	Ready-to-use	4 °C

14. The temperature for Wash I and Wash II is different. Wash I is at 25 °C and Wash II is at 68 °C.

15. Detection reagent setup can be found in Table 6.

16. Blocking buffer is made with 15 mL of 10× blocking buffer (which can be *saved at* −20 °C), 15 mL of 10× maleic acid, and 120 mL water. Blocking buffer needs to be made freshly.

17. Anti-DIG buffer is made by adding 3 μL anti-DIG-AP conjugate into 30 mL blocking buffer.

18. The plastic sheet is big enough to cover three times the size the membrane.

19. Stripping and rehybridization only apply to probes that are designed to hybridize to genomic DNAs digested with the same restriction enzyme.

Acknowledgments

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Use of Multicolor Flow Cytometry for Isolation of Specific Cell Populations Deriving from Differentiated Human Embryonic Stem Cells

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Abstract

Flow Cytometry-Sorting (FCM-Sorting) is a technique commonly used to identify and isolate specific types of cells from a heterogeneous population of live cells. Here we describe a multicolor flow cytometry technique that uses five distinct cell surface antigens to isolate four live populations with different surface antigen profiles. These profiles were used to help distinguishing between neural and nonneural (the lens) ectoderm derivatives within a highly heterogenous population of differentiating human embryonic stem cells (hESC).

Keywords: Flow cytometry, hESC, Differentiation, Fluorochromes, Antibodies, Sorting-analysis

1 Introduction

Human embryonic stem cells lines were originally derived from human blastocysts and shown to be pluripotent, therefore capable of generating tissues that derive from all three germ layers (1). Because of their origin, differentiated hESC cultures are heterogeneous, nevertheless analysis of specific cell populations requires the isolation of the cells of interest. FCM provides the researcher with a tool to analyze cell composition in differentiating hESC cultures and allows the isolation of target cells. In a FCM machine a suspension of cells passes through a laser beam that reads intrinsic characteristics like size, complexity, and autofluorescence. In addition to these parameters, this machine can analyze cells that express an internal fluorescent reporter, have incorporated a fluorescent dye (as a probe) or have been stained with fluorochrome-conjugated antibodies (2). Modern flow cytometers are high-speed machines, analyzing cells at >100,000 cells/s and sorting at >30,000 cells/s. The fluorescence measurement is scalable with the addition of multiple laser lines, up to 10 different wavelengths from Ultraviolet (UV) to Infrared (IR). The fluorescence is measured by up to 20 detectors using bandpass filters spectrally matched to the target fluorochromes. Thanks to the power of this instrumentation

FCM-sorting can identify and isolate groups of cells that express the same, preselected profile of multiple fluorescent antigens (3, 4). The method we describe here relies on the ability of antibodies to specifically recognize epitopes on the cells surface. Therefore, since no intracellular epitopes are examined, permeabilization of cells is not required, allowing the collection of live cells. The primary antibody (herein referred to as: pAb) can be either directly conjugated to a fluorochrome or it can be recognized by a fluorochrome-conjugated secondary antibody (herein referred to as: sAb) that must recognize the pAb with high specificity. Primary antibodies directly conjugated to fluorochromes are extremely useful to simplify the procedure of cell immunolabelling in multicolor FCM-Sorting applications. When sAbs are used, they should be conjugated to a fluorochrome and specifically recognize the pAbs species or isotype (alternatively a biotin–streptavidin bond can be used to link a pAb to a fluorochrome). It is important to use fluorochromes with minimum spectral overlap into other detectors.

In this chapter we describe the use of multiple pAbs directed against five different surface molecules for the identification and isolation of four different live cell populations, with distinct surface antigen profiles. We show how the specific selection of the target antigens allows the discrimination between neural and nonneural (lens) types of ectoderm derivatives, formed during early differentiation of hESC. We discuss the limitation inherent to the use of a sAb together with multiple pAbs isotypes. Finally, we explain the rationale followed in setting up the sorting gates for selection of the desired population types by describing the gating strategies used during post-sort software (FlowJo) analysis.

2   Materials

2.1 Solutions and Tools for Preparation and Immunostaining of the Cells

1. DPBS solution (Dulbecco's Phosphate Buffer Saline, without Calcium and Magnesium).
2. HBSS (Hanks' Balanced Salt Solution modified, with phenol red, without calcium and magnesium, liquid).
3. Trypsin (0.25 % Trypsin-0.02 % EDTA Solution).
4. DMEM/F-12 (Dulbecco's Modified Eagle Medium/ F-12).
5. FBS (Fetal Bovine Serum).
6. Sorting Buffer: for a tot volume of 100 ml mix 50 ml EDTA (0.02 %), 47 ml PBS, 2 ml FBS or FCS (Fetal Calf Serum), 1 ml Penicillin/Streptomycin solution (100×) and pass through a 0.22 μm syringe filter. Work in sterile conditions in a biosafety cabinet. Store it at 4 °C.
7. Trypan Blue: 0.4 % in PBS.
8. Antibodies: Table 1.

Table 1
Components in each preparation tube

Tube	Label	Cells	Primary antibody	Secondary antibody	Target antigen	Stock concentration	Final dilution of pAb[a]	Final dilution of sAb[a]
Sample (tube1)	Sample	+	Anti-p75, mouse IgG1 -PE[b]		Human p75 CD271	100 µg	1:100	
			Anti-c-met, mouse IgG1-APC[b]		Human c-met (HGFR)	10 µg/ml	1:20	
			Anti-CD44, rat IgG2b-APC/Cy7[b]		Human CD44	200 µg/ml	1:100	
			Anti-HNK-1, mouse IgM	Goat anti-mouse IgM-AF488	Human CD57 (HNK-1)	200 µg/ml	1:150	
			Anti-CD15, mouse IgM-PB[b]		Human CD15	Unknown	1:133	
Control (tube 2)	Unstained	+						
Control (tube 3)	PE	+	Anti-p75, mouse IgG1 -PE[b]					
Control (tube 4)	APC	+	anti-c-Met, mouse IgG1-APC[b]					
Control (tube 5)	APC-Cy7	+	Anti-CD44, rat IgG2b-APC/Cy7[b]					
Control (tube 6)	AF488	+	Anti-HNK-1, mouse IgM	Goat anti-mouse IgM-AF488		2 mg/ml (for sAb)		1:400
Control (tube 7)	PB	+	Anti-CD15, mouse IgM-PB[b]					
Control FMO (tube 8)	-APC	+	All primary Ab except anti-c-Met-APC	Goat anti-mouse IgM-AF488				
Control FMO (tube 9)	-AF488	+	All primary Ab except anti-HNK-1					

Control FMO (tube 8 and 9) are suggested controls if needed, *PE* phycoeritrin, *APC* allophycocynin, *AF488* Alexa Fluor 488, *PB* pacific blue
[a]µl stock antibody/µl final volume of incubation buffer
[b]Directly conjugated antibody

9. 18 Gauge (18 G), 40 mm (length) and 21 Gauge (21 G), 40 mm (length) needles.

10. 20 ml syringe.

11. 40 μm cell strainer (for 50 ml tube diameter).

12. 15 and 50 ml tubes. 5 ml, round-bottom, polypropylene (12 × 75 mm) tubes.

13. Neubauer Cell Counting Chamber and Counter Tally.

14. Twister Shaker Platform (place it at 4 °C).

2.2 Solutions and Tools for Collection of Live Cells

1. ITS medium: 985 ml of MilliQ distilled water, DMEM/F12 powder (Gibco, one bag for 1 l medium), 1.55 g D-glucose, 2.438 g NaHCO₃, 50 mg human apo-transferrin, 5 mg human insulin (dissolved in 5 ml of 10 mM NaOH), 60 μl Na Selenite (0.5 mM stock in distilled water), 10 ml Penicillin/Streptomycin (100×). Assemble the component in a beaker with magnetic stirring, transfer the solution under a biosafety cabinet, filter it through a 0.22 μm filter and cover the bottle with aluminum foil to protect from light. Store at 4 °C.

2. Y27632 Rock Inhibitor (10 μM final).

3. FGF2 (basic fibroblast growth factor).

4. EGF (epidermal growth factor).

5. Table centrifuge with swing bucket rotor and adapters for both 5 ml round bottom and 15 ml tubes.

2.3 Equipment and Post-sorting Analysis Software

1. BD Influx (five lasers) flow cytometry sorter.

2. FlowJo software.

3   Methods

The following procedure can be applied to differentiating cells growing in adherent conditions in one 6 cm cell culture dish. The starting culture can be scaled up if the populations of interest are known to be exiguous. Work is done under a biosafety cabinet in sterile conditions. Steps are carried out at room temperature (RT) unless otherwise specified.

3.1 Preparation of Cells for Immunostaining in Suspension

1. Remove medium from the dish. Wash the adherent cells once with 3 ml PBS. Aspirate it. Add 2 ml HBSS and leave at room temperature (RT) for 20–30 min. Aspirate it (see Note 1).

2. Add 0.8–1 ml Trypsin solution to the cells, transfer at 37 °C/5 % CO₂ and incubate for 5 min. Under a biosafety cabinet use a P1000 pipetman (set at 1,000 μl) to gently aspirate and release the suspension of cells a few times onto the bottom surface of the dish until all cells are detached. Immediately add 3 ml of a

solution containing FBS to the cell suspension to inactivate the trypsin. Transfer the suspension of cells into one 50 ml sterile polypropylene tube. Complete collection of the cells from the dish by washing with 2–3 ml PBS and collect them into the same 50 ml tube (see Note 2).

3. To ensure single cell suspension, pass the solution with cells through a sterile syringe equipped with an 18 G needle. Repeat by passing the cells four times through the same syringe with a 21 G needle. Place a 40 μm cell strainer over a new sterile 50 ml polypropylene tube. Filter the entire volume of the cell suspension. Finish collecting the cells from the old tube by using 5 ml PBS and pass it through the strainer into the same new tube (see Note 3).

4. Centrifuge the cells collected in the 50 ml tube at $310 \times g$ for 10 min at RT.

5. Prepare new 15 ml polypropylene tubes in which you will distribute the pelleted cells and label them as indicated in Table 1. The appropriate control tubes need to be prepared at this time (see Note 4).

6. After centrifugation, discard the supernatant using a glass capillary pipette connected to vacuum suction. Tap the pellet to loosen it. Using a P1000 pipetman resuspend the pellet in 800 μl of 4 °C-cold Sorting Buffer (see Note 5). Generate a homogeneous-looking cell suspension by gently pipetting the cells up–down. Store the cell suspension on ice.

7. Count cells in the suspension by diluting an aliquot of these cells (e.g. 10 μl) in Trypan Blue solution (e.g. 190 μl) and mix well in a 1.5 ml tube. Load 10 μl of this cell suspension in a hemocytometer chamber. Cover the suspension with the coverslip being careful not to generate bubbles. Count the total number of live cells (that do not internalize the Trypan Blue dye) in the four larger quadrants at the four angles of the chamber grid ($=n$), under a $10\times$ magnification objective. Calculate $N =$ total number of cells/ml (see Note 6).

8. Distribute $1–5 \times 10^5$ cells in each control tube (15 ml tubes, see Note 7) and adjust the final volume to 300 μl with 4 °C-cold Sorting Buffer. Adjust the final volume of the sample tube to 800 μl with Sorting Buffer (transfer it to a 15 ml tube). The cells of the control (tube 2, unstained) are ready for FCM analysis; transfer them to a 5 ml, round-bottom, polypropylene tube and store them on ice until the remaining cells will be ready.

9. Add the unconjugated pAbs at the concentration indicated in Table 1 (see Note 8) to the control (tube 6) and to sample (tube 1) and to the FMO control (tube 8, if this control is prepared). Incubate cells with the primary antibody for 30 min at 4 °C (cold room) positioning the tubes on a rocking platform set to produce a gentle movement of the cell suspension along the tube (see Note 8).

10. Add 5 and 12 ml PBS in the control (tube 6) and sample (tube 1) respectively to wash away the unbound antibody. Centrifuge tubes at $310 \times g$ for 5 min (see Note 9). After centrifugation, carefully remove all the supernatant paying attention not to disturb the pellet. Resuspend the cell pellets of the control (tube 6) and sample (tube1) in 400 μl and 800 μl 4 °C-cold Sorting Buffer respectively. Use a P1000 pipetman and gently resuspend the pellets to obtain a visually homogenous cell suspension.

11. Add the AF488-conjugated sAb to the cell suspension in control (tube 6) and sample (tube 1) at the concentration indicated in Table 1. Minimize the exposure to light of the fluorochrome-conjugated antibodies (see Note 10). Incubate the cells for 30 min at 4 °C on a rocking platform.

12. Wash away the unbound sAb by adding 5 and 12 ml PBS to the control (tube 6) and sample (tube 1) respectively. Centrifuge the tubes at $310 \times g$ for 5 min. Aspirate the supernatant from the two tubes as much as possible, paying attention not to disturb the pellet. Repeat the wash for the sample (tube 1). Resuspend the cell pellets of the control (tube 6) and sample (tube 1) in 300 and 800 μl 4 °C-cold Sorting Buffer respectively. The cells in control (tube 6) are ready for analysis, transfer them to a 5 ml, round-bottom tube, keep them on ice, in the dark, until when the remaining cells will be ready.

13. Add the directly conjugated pAbs to all the remaining control tubes (3–5, 7 and 8, 9 if used) and to the sample (tube 1) at the dilution indicated in Table 1. Protect tubes form the light and incubate the cells for 30 min on the rocking platform at 4 °C.

14. Add 5 ml (to the control tubes) and 13 ml PBS (to the sample, tube 1) to wash away unbound antibodies and centrifuge them at $310 \times g$ for 5 min. Discard the supernatant and resuspend the cell pellets in 400 μl (control tubes) and 2 ml (sample, tube 1) of 4 °C-cold Sorting Buffer (see Note 11). Using a P1000 pipetman set to maximum volume, pipet up–down the sample cell suspension to break large, loosely formed, cell aggregates. Filter this suspension through a 40 μm cell strainer positioned (kept in place manually) directly on a 5 ml, round-bottom tube into which the cell suspension will be finally collected (see Note 11). Transfer all cell suspensions of the control tubes into 5 ml round-bottom tubes. Put all tubes in ice and keep them protected from light. Prepare the medium (ITS) necessary for collection of the desired subpopulation of cells already containing growth and survival factors (e.g. Rock Inhibitor 10 μM, FGF2 10 ng/ml, EGF 20 ng/ml). Put 1 ml of ITS medium in four 5 ml, round-bottom tubes, for cell collection. Growth factors for specific subpopulations can be added to the tube in which that population will be collected. Keep all capped tubes on ice.

3.2 Sorting Settings and Strategy

The control tubes are run in the following order. Tube 2 (unstained), tube 7 (PB), tube 6 (AF488), tube 3 (PE), tube 4 (APC), tube 5 (APC-Cy7), then followed by tube 1 (sample). Gating Strategy: dead cells and debris are excluded from further analysis by plotting FSC (forward scatter) vs. SSC (side scatter) signals, cells in the gate (called "live cells") in Fig. 1a are subsequently analyzed in a FSC vs. FSC Int. plot. From this second plot, cells in doublets or triplets (with smaller FSC value) are excluded by selecting the cells gated as in Fig. 1b. Only these "single cells" are further analyzed in each tube. In our experiments we collect the four populations indicated in Table 2.

1. A gate is defined around the single cells of tube 2 (unstained) in dot plots for the following combinations of channels: APC vs. PE (combination a, Fig. 1c), APC-Cy7 vs. AF488 (combination b, Fig. 1d), PB vs. AF488 (combination c, Fig. 1e) (see Note 12).
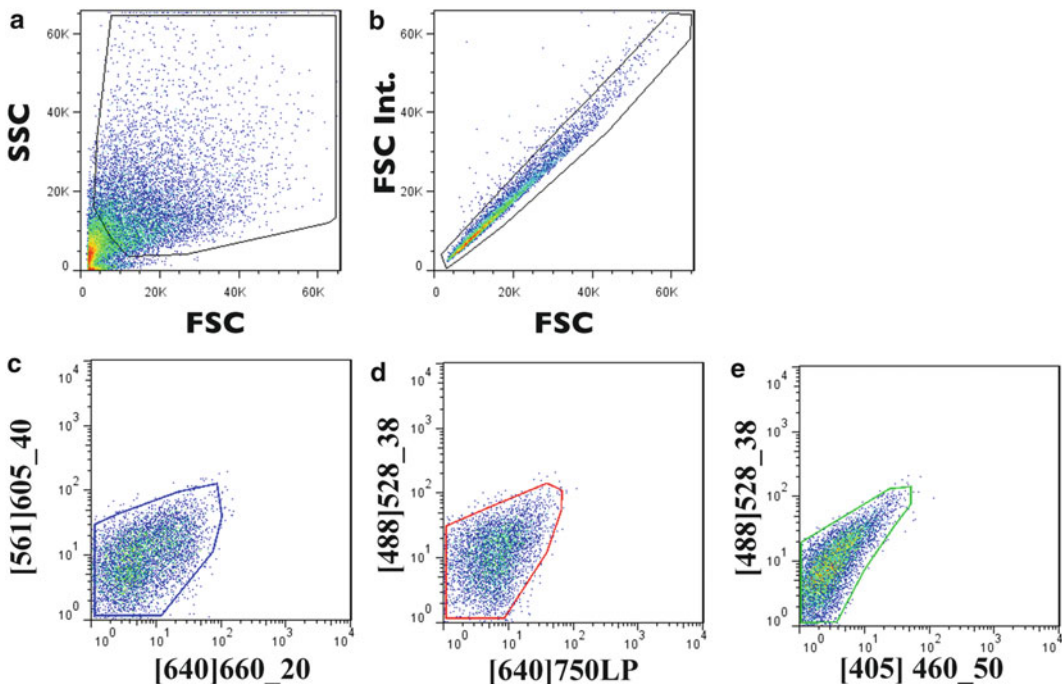
Fig. 1 Analysis of the unstained cells. (**a**) definition of "live cells" gate (axes: FSC vs. SSC). (**b**) definition of "single cells" gate as subpopulation of "live cells" gate (axes: FSC vs. FSC Intensity). (**c–e**) definition of the "negative cells" gates in the following channels; (**c**): APC = [640]660_20 vs. PE = [561]605_40 (called "combination a." (**d**): APC-Cy7 = [640]750LP vs. AF488 = [488]528_38 (called "combination b"). (**e**) PB [405] 460_50 vs. AF488 = [488]528_38 (called "combination c")

Table 2
Surface profile (by fluorochrome) of the sorted populations

	Populations			
Fluorochromes	1	2	3	4
APC-Cy7	+	+	−	−
APC	−	+	+	−
PE	−	−	−	−
AF488	−	−	−	+
PB	−	−	−	+
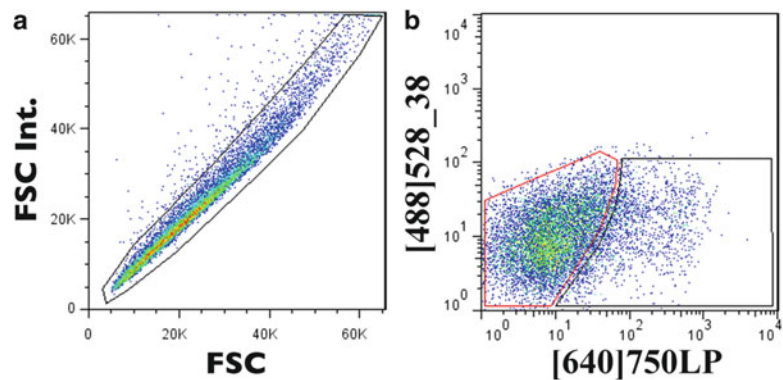
Fig. 2 Analysis of cells in a single color control tube (example: APC-Cy7). (**a**) definition of "single cells" gate as subpopulation of "live cells" gate (not shown). (**b**) definition of negative cells (*red gate*) and positive cells (*black gate*) in the combination of channels that include APC-Cy7 = [640]750LP ("combination b")

2. Analyze the cells from the "single cells" gate of each single-color control tube (tubes 3–7) in a dot plot, the axis of which includes the fluorochrome of that particular single color tube (e.g. Fig. 2b). Draw the gate that defines the boundary between positive and negative cells for that fluorochrome (Fig. 2b; see Note 13).

3. Now analyze the sample cells (of tube 1) defining first the "live cells" and their "single cells" subpopulation (as shown above), then using a sequence of dot plots with the fluorochrome combinations used before (combinations a, b, c). In each plot define the gates only for the cells with the desired (indicated in Table 2) fluorochrome profile (Fig. 3, see Note 14). After collection, cells can be further analyzed with different techniques. Upon further molecular analysis the cells of population 3 resulted to be human eye lens cells (5).
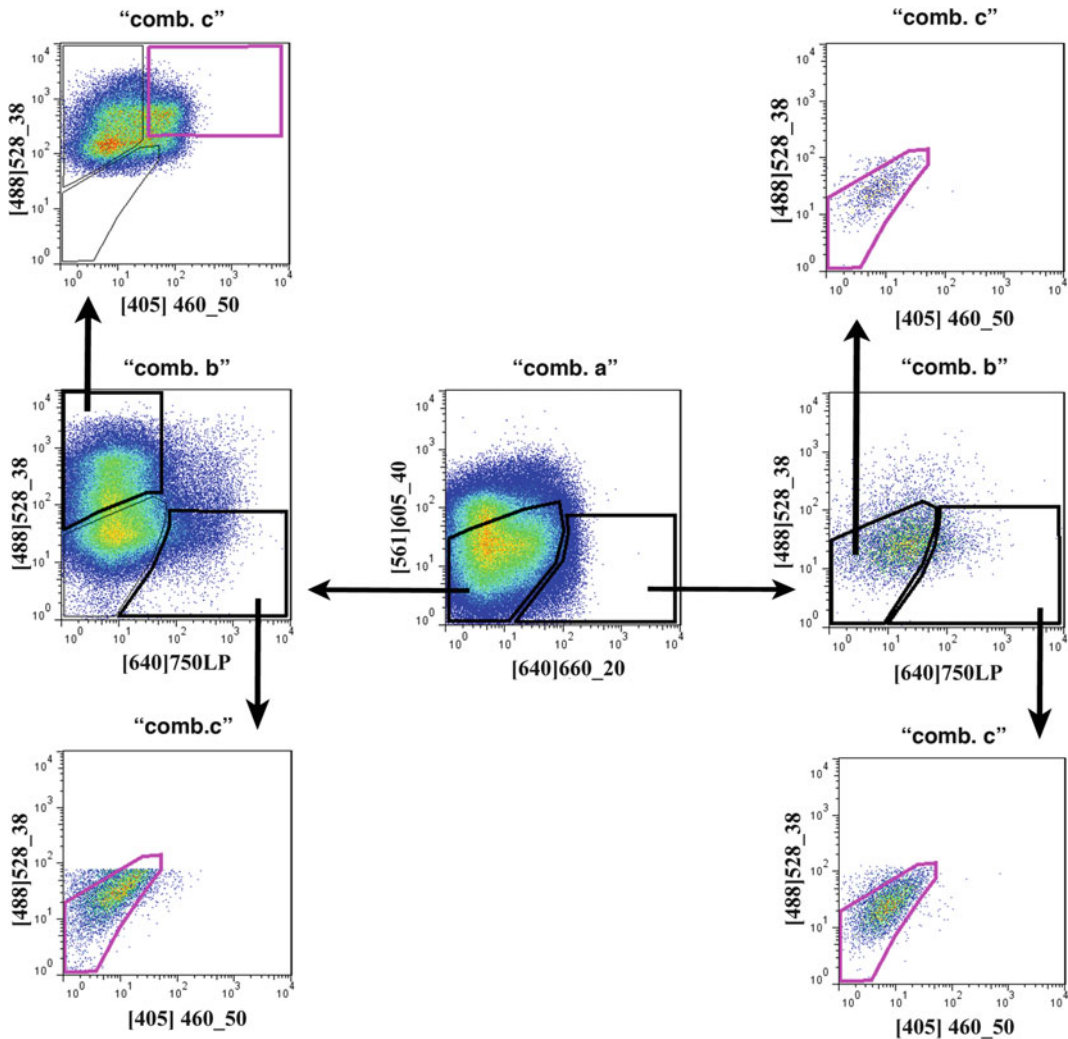
Fig. 3 Analysis of cells in the sample tube. The flow of analysis goes from the central panel ("comb. a"= combination a) following the black arrows to the side panels ("comb. b" and then "comb. c"). Cells in the *central panel* represent the single cells that are a subset of live cells (analyzed as in Fig. 1). *Black arrows* indicate how cells from each *black gate* are further analyzed. Cells within the *pink gates* represent the desired final subpopulations indicated in Table 2

4   Notes

1. The PBS wash helps removal of dead cells and debris from the top surface of the cells facilitating the action of the HBSS solution, which, by depleting Ca^{2+} and Mg^{2+} ions, helps disrupting the interaction among cell adhesion molecules. Application of this solution is sometimes sufficient to release cells, but tissues in advanced stage of differentiation present more

complex cell adhesion interactions that require enzymatic treatment to dissociate.

2. For cell sorting any tissue should be dissociated to a single-cells suspension. If the action of enzymes is necessary, particular care must be taken in choosing the enzyme because the extracellular portion of some cell membrane molecules may be completely digested, therefore eliminating a potential target epitope of the pAbs (6). Different "gentler" enzymes are now commercially available (e.g. TrypLE Express, TypLE Select, Accutase), it is advisable to experimentally test which enzymes are the most effective in disaggregating the cells with minimum damage. Incubation with Trypsin should be protracted for the shortest time possible, it may be helpful to follow the detachment of the cells by eye or under 5× magnification at 2 min incubation intervals. Try not to protract trypsin digestion beyond 10 min. A fraction of cells is usually lost due to harsh treatment with trypsin, which should be used only on though-to-dissociate tissue types. After mechanical dissociation through the pipette, mix well the FBS solution (3 ml DMEM/F12 containing 10 % FBS) with the cell suspension to help inactivation of the enzyme.

3. Depending on the total volume of the cell suspension, choose syringes with the appropriate capacity so that the entire volume of cells can be aspirated at once. Needles with different diameters are commercially available, use a larger needle diameter first (to start disrupting the larger clumps) and then apply a smaller needle (to complete a finer cell cluster disruption). At this point most cells should be in single-cells condition. Inevitably some cluster remains, but they will be filtered out using the strainer.

4. In multicolor FCM experiments it is necessary to include multiple control tubes, which include the following: (1) unstained control, (2) single color controls, and (3) Fluorescence Minus One (FMO) controls. The unstained control is used to setup the flow cytometer. This control defines the background or autofluorescence of the cells thus setting the boundary for the "negative" cells (Fig. 1). Single color controls: each fluorochrome used will have a separately stained control. This will be used to compensate for spectral overlap into other detectors and will aid in defining gating boundaries between "positive" and "negative" cells (an example in Fig. 2). Although not performed in our experiment, if upon analysis of fluorescence emission in any channel it results difficult to precisely set the boundary between positive and negative cells (because of a weak fluorescence signal) it can be useful to add an FMO control tube, as indicated in Table 1. In this tube all fluorochromes are added with the exception of

the one that is in question. During analysis it will be clearer at what level to set the boundary for the "negative" cells in that channel (3). Possible FMO controls for this experiment are indicated in the last two rows in Table 1, they would help defining the c-Met+ and HNK-1+ cells during gates definition in those channels.

5. The initial volume in which to resuspend the cell pellet can be chosen on the bases of knowledge, from previous experiments, of the expected number of cells that can be obtained from one 6 cm dish of differentiating hESC. In our procedure $20–30 \times 10^6$ cells were usually obtained from one dish. After counting the cells we adjusted the volume of cell suspension to have $2.5–3.75 \times 10^6$ cells in 100 μl Sorting Buffer for the staining incubation of the "sample" tube.

6. It is advisable to start from a low dilution factor, e.g. 10 μl cell suspension in 90 μl Trypan Blue, which corresponds to a dilution factor of 10. If the number of cells in the quadrants is too high to clearly count the single cells, further dilute the suspension. The number of cells/ml in your initial suspension can be calculated by the formula: $N = (n \times 2{,}500 \times dilution factor)$. Counting the cells helps evaluating how much of the initial suspension to distribute in the control tubes and how many cells are present in the sample tube/s.

7. A minimum of 1×10^5 cells is usually sufficient in the control tubes to provide a clear signal for gates definition. However, to help formation of a well-compacted pellet (necessary to remove most of the antibody-containing supernatant after the following centrifugations) distribute about 2.5×10^5 cells in each control tube (up to a maximum of 5×10^5 cells).

8. The five target antigens were selected as follows. The CD271, CD15, and HNK-1 molecules are known to be expressed on neural progenitor and nervous system cells (7, 8), therefore they were used for negative selection of the neural component within populations 1–3 (Table 2). The c-Met and CD44 antigens are instead expressed on human lens cells (a nonneural ectoderm derivative) (9, 10) and used for positive selection of populations with lens fate potential. Most pAbs used are directly conjugated to a fluorochrome, except for the anti-HNK-1 antibody, for which the use of a fluorochrome-conjugated sAb was necessary. Since this sAb may cross-react with the anti-CD15 pAb (because directed against the same pAb's isotype) the staining protocol was modified, as described below, to minimize cross-reaction. The final concentration of each pAb (Table 1) should be defined experimentally. The AF488-conjugated sAb is directed against any mouse IgM pAb therefore it may cross-react with the anti-CD15 pAb (also an IgM). To minimize the cross-reaction we incubated the cells, in control and sample tubes, first with

the anti-HNK-1 antibody alone, and then, after the wash, with the AF488-conjugated sAb. The unbound sAb was then washed away twice with PBS before incubation with the remaining pAbs. In absence of a rocking platform, incubation with the pAbs can be performed by keeping the cells on ice (protected from light) for 30 min, providing manual agitation every 5–10 min to avoid cell sinking. It is possible to spectrally distinguish the emissions of propidium iodide (PI) and phyco-erythrin (PE) (11), therefore PI could be added to the sample cell suspension to help the selection of alive cells that exclude this dye. To avoid additional chemical stress to our cells of interest we avoided the use of PI.

9. If working with a large number of cells, a 50 ml tube may be used for the sample cells; in this case the centrifugation time can be prolonged to 10 min. If cells still appear not well pelleted, centrifugation speed may be increased (e.g. to $350 \times g$).

10. To minimize the exposure to light turn off the light in the biosafety cabinet and cover the ice buckets with the lid.

11. An ideal final cell suspension is 10×10^6 cells/ml, thus defining the volume in which to resuspend the pelleted cells. However, a too concentrated cell suspension may favor formation of small cell aggregates that will be excluded by the sorting through the gating strategy, resulting in a decreased number of effectively sorted cells. Additionally, cell aggregates may clog the sorter nozzle incurring unwanted experimental delay. On the other hand, the speed of sorting may be increased if the cell sample is not too diluted. The speed of sorting is also affected by the nozzle size ("smaller" nozzle size generates more drops, accelerating the sorting speed compared to larger nozzle sizes). However, the nozzle size should be chosen in relation to the size of the cells. Ultimately, the optimal cell concentration will be determined empirically. The small number of cells in the control tubes usually does not form large aggregates; therefore, if the final cell suspension is visually homogeneous, filtration of these cells can be avoided. For filtration of cell suspensions that are not too dense, 5 ml round bottom tubes, equipped with filter caps, are commercially available. However, for dense or large volume of cells we found it faster and equally effective to use 40 μm filter strainers (for 50 ml tubes) on 5 ml tubes. The cells of interest are directly collected in media supplemented with factors that support their proliferation and survival (12–14).

12. Analyze the fluorescent signals by using dot plots. Any combination of two channels at a time can be chosen for analysis of the signals in each dot plot. Keeping in mind the fluorescent profile of the desired final populations (indicated in Table 2) each dot plot will provide information about

which cells are negative (or positive) for each specific fluorochrome.

13. To distinguish a "positive" fluorescence signal derived from correctly immuno-labelled cells from a "negative" signal derived from background fluorescence, we compared the signal detected in each single color control tube to the signal given by the unstained cells in that specific fluorochrome channel. This comparison is made by transferring the gate defined on the unstained cells for each fluorochrome channel (as in point 1) to the dot plot of each single color control.

14. The cells in each gate defined in dot plot "comb.a" will be separately analyzed in dot plots "comb.b" and cells from each gate in dot plots "comb.b" will be analyzed in dot plots "comb. c." To define the boundaries between positive and negative cells for each fluorochrome use the gates defined for the unstained and single color control cells. In using these previously defined gates, the investigator can apply at his/her own discretion different levels of "stringency," that is how close a new gate will be to the gate that defines the "negative" cells.

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Definitive Endoderm Differentiation of Human Embryonic Stem Cells Combined with Selective Elimination of Undifferentiated Cells by Methionine Deprivation

Tomonori Tsuyama, Nobuaki Shiraki, and Shoen Kume

Abstract

Human embryonic stem cells (ESCs) show a characteristic feature in that they are highly dependent on methionine metabolism. Undifferentiated human ESCs cannot survive under the condition that methionine is deprived from culture medium. We describe here a procedure for definitive endoderm differentiation from human ESCs, in which human ESCs are subject to 10 days (d) differentiation combined with methionine deprivation between differentiation day (d) 8 to d10. Methionine deprivation results in elimination of undifferentiated cells from the culture with no significant loss of definitive endoderm cells, as compared to those cultured under complete condition throughout the whole culture period.

Keywords: Methionine deprivation, Endoderm differentiation, M15 cells, Selective elimination, Undifferentiated cells, Metabolic difference

1 Introduction

Human embryonic stem cells and/or induced pluripotent stem cells (hESCs/iPSCs) have been proposed as a cell source for drug discovery and cell replacement therapy. To this end, we established several protocols for stepwise differentiation of hESCs/iPSCs towards the definitive endoderm lineage (namely, the pancreas, liver, and intestine) (1–8).

One concern with hESCs/iPSCs-based approach is the differences in differentiation potentials among cell lines (9, 10). Certain cell lines are refractory to definitive endoderm differentiation and a substantial population of undifferentiated cells remain although other cell lines achieve high efficiencies of endoderm differentiation with the same differentiation protocol. The persistence of undifferentiated cells is undesirable because these cells might be inhibitory for further differentiation and might form tumor after transplantation.

Human ESCs/iPSCs are in a higher flux methionine metabolism, compared to definitive endoderm (11). Utilizing this metabolic difference, we developed a protocol to eliminate the remaining undifferentiated cells (11).

This protocol is based on our previous culture system (coculture with M15 feeder cells in RPMI supplemented with 100 ng/ml activin A for 10 d) (4) with a slight modification in using methionine-deprived medium from d8 to d10. A protocol without using M15 cells is also described.

2   Materials

1. M15 cells (ECACC cell no. 95102517).

2. Culture Dish and Plate (60-mm, BD falcon, 353004)(90-mm, Nunc, 150350)(150-mm, Nunc, 168381)(96-well plate, Corning, 3595).

3. PBS (*see* **Note 1**).

4. 0.05 % trypsin/EDTA (Invitrogen, 25300-062), 0.25 % Trypsin/EDTA (Invitrogen, 25200-072).

5. EF medium.

DMEM (Invitrogen, 11995-075)	500 ml
FBS (Hyclone)	58 ml
Penicillin and streptomycin (PS: Nacalai tesque, 26252-94) (*see* **Note 2**)	5.8 ml
L-Glutamine (L-Gln; Nacalai tesque, 16948-04) (*see* **Note 2**)	5.8 ml

6. 2× Freeze solution.

EF medium	28 ml
DMSO (Sigma, D2650)	10 ml
FBS (Hyclone)	2 ml

7. Mitomycin C solution.

Dissolve mitomycin C (2 mg, Sigma, M4287) in 2 ml PBS.

8. Mitomycin C-containing medium.

EF medium	200 ml
Mitomycin C solution (Final concentration: 10 μg per ml)	2 ml

9. Human ESCs medium.

Reproff (ReproCELL, RCHEMD004)	500 ml
PS (Nacalai Tesque, 26252-94) (*see* **Note 2**)	5 ml

10. Supplements for human ESCs medium.
bFGF (Peprotech, 100-18B-2).

Stock solution at 5 μg/ml in 0.1 % (w/v) BSA/PBS. Aliquot into 100 μl and store at −80 °C. Once thawed, keep at 4 °C. Add to human ESCs medium at a final concentration of 5 ng/ml.

11. Y27632 (Wako, 253-00513) (*see* **Note 6**).

12. Matrigel (BD, 354234) (*see* **Note 7**).

13. Endoderm Differentiation basal Medium 1 (store at 4 °C).

RPMI 1640 medium (Invitrogen, 11875-093)	500 ml
PS (Nacalai tesque, 26252-94) (*see* **Note 2**)	5 ml
L-Glutamine (Nacalai tesque, 16948-04) (*see* **Note 2**)	5 ml
NEAA (Invitrogen, 11140-050) (*see* **Note 2**)	5 ml
0.1 M ME (*see* **Note 3**)	500 μl

14. Endoderm Differentiation Basal Medium 2 (store at 4 °C).

Methionine-deprived medium (Ajinomoto, provided upon requests) (or Cell Science & Technology Institute, a custom-made medium)	500 ml
PS (Nacalai tesque, 26252-94) (*see* **Note 2**)	5 ml
L-Gln (Nacalai tesque, 16948-04) (*see* **Note 2**)	5 ml
NEAA (Invitrogen, 11140-050) (*see* **Note 2**)	5 ml
0.1 M ME (*see* **Note 3**)	500 μl

15. Supplements for endoderm differentiation Medium (store at 4 °C).
Activin (R&D, 338-AC).
Stock solution at 100 μg/ml in 0.1 % (w/v) BSA/PBS. Aliquot into 100 μl and store at −80 °C. Once thawed, keep at 4 °C. Add to endoderm differentiation medium at a final concentration of 100 ng/ml.
B27 supplement (Invitrogen, 17504-044).
Stock solution at 100 % (50×). Aliquot into 500 μl and store at −20 °C. Once thawed, keep at 4 °C. Add to endoderm differentiation medium at a final concentration of 2 % (v/v, 1×).

3   Methods

3.1   Preparation of Mitomycin C-Treated M15 Cells (MMC-M15 Cells)

3.1.1   Thawing M15 Cells

1. Put a vial of frozen M15 stock into 37 °C water bath until most cells are thawed.

2. Transfer cell suspension to 15 ml tube pre-added with 4 ml EF medium.

3. Spin down at 180 × *g* for 5 min.

4. Resuspend the pellets with 10 ml EF medium

5. Transfer cell suspension into 90-mm dish

6. Incubate at 37 °C under 5 % CO_2.

3.1.2 Passage of M15 Cells

1. At full confluence, remove the medium.

2. Rinse with PBS.

3. Add 0.05 % trypsin/EDTA (1 ml per 90-mm dish, 3 ml per 150-mm dish) and incubate for 5 min at 37 °C under 5 % CO_2.

4. Add EF medium into the M15 cells dish (4 ml per 90-mm dish, 6 ml per 150-mm dish), suspend cells by gently pipetting, and transfer cell suspension into 15 ml tube or 50 ml tube.

5. Spin down at 180 × *g* for 5 min.

6. Resuspend the pellets with an appropriate amount of EF medium.

7. Seed the cells at 1.5×10^6 cells per 150-mm dish

8. Incubate at 37 °C under 5 % CO_2 until they reach confluence.

3.1.3 Mitomycin C-Inactivation of M15 Cells

1. Remove the medium.

2. Add mitomycin C-containing medium, and incubate for 2 h at 37 °C under 5 % CO_2.

3. Remove the mitomycin C-containing medium.

4. Rinse with PBS twice

5. Add 3 ml of 0.05 % trypsin/EDTA and incubate for 5 min at 37 °C under 5 % CO_2.

6. Add 5 ml EF medium, suspend cells by P1000 pipetting, and transfer cell suspension into 50-ml tubes.

7. Spin down at 180 × *g* for 5 min.

8. Resuspend the pellets with EF medium to the concentration at 2×10^7 cells/ml.

9. Add equal volume 2× freeze solution and mix gently.

10. Transfer 1 ml of cell suspension into cryovials.

11. Put cryovials into Nalgene controlled-rate freezer box and then put the box into −80 °C freezer.

12. On the next day, transfer the vials of frozen MMC-M15 cells into −150 °C freezer for long-term storage.

3.2 Preparation of Gelatin-Coat Plates

1. Add 50 μl of 0.1 % gelatin solution into 96-wells plate.

2. Incubate at 37 °C for 2 h (*see* **Note 4**).

3. Remove gelatin solution.

4. Add 100 μl EF medium into 96-well gelatin-coated plate.

3.3 Preparation of MMC-Treated M15 Feeder Plates

1. Take a vial of MMC-M15 cells from $-150\ ^{\circ}C$ freezer and put into $37\ ^{\circ}C$ water bath until most cells are thawed.

2. Transfer MMC-M15 cells into a 15 ml tube pre-added with 4 ml EF medium.

3. Spin down at $180 \times g$ for 5 min.

4. Resuspend the pellet with EF medium to the concentration at 4.0×10^5 cells/ml.

5. Add 100 μl MMC-M15 cell suspension onto 96-well gelatin-coated plates pre-added with 100 μl EF medium (Section 3.2).

6. Incubate at $37\ ^{\circ}C$ under 5 % CO_2.

7. On the next day, MMC-M15 cells are ready to be used as feeders for human ESCs differentiation.

3.4 Plating and Differentiation of Human ESCs (See Note 5)

1. Remove human ESCs medium.

2. Rinse with PBS.

3. Add 0.25 % trypsin/EDTA and incubate at $37\ ^{\circ}C$ for 5 min.

4. Remove 0.25 % trypsin/EDTA.

5. Add 2 ml EF medium and suspend the cells by pipetting with a P1000 pipet.

6. Add 3 ml EF medium and transfer 5 ml of cells suspension into 15 ml tube.

7. Spin down at $180 \times g$ for 5 min.

8. Resuspend the pellet with human ESCs medium supplemented with 10 μM Y27632 to the concentration at 1×10^5 cells/ml.

9. Remove EF medium from the MMC-M15 cells plates (Section 3.3) and add 100 μl fresh human ESCs medium with 10 μM Y27632 into MMC-M15 cells plates.

10. Add 100 μl of cell suspension into MMC-M15 96-well plate pre-added with 100 μl of human ESCs medium.

11. Incubate at $37\ ^{\circ}C$ under 5 % CO_2.

12. On the next day, remove human ESCs medium.

13. Rinse with PBS.

14. Change medium with fresh endoderm differentiation medium 1 supplemented with both Activin and B27 at day 0, 2, 4, 6 from the onset of differentiation.

15. Switch the medium to endoderm differentiation medium 2 supplemented with both Activin and B27 at day 8 from the onset of differentiation and culture cells for 2 days (*see* **Note 8**).

3.5 Plating and Differentiation of Human ESCs (Optional, Feeder-Free System) (See Note 5)

1. Remove human ESCs medium.

2. Rinse with PBS.

3. Add 0.25 % trypsin/EDTA and incubate at 37 °C for 5 min.

4. Remove 0.25 % trypsin/EDTA.

5. Add 2 ml EF medium and suspend the cells by pipetting with a P1000 pipet.

6. Add 3 ml EF medium and transfer 5 ml of cells suspension into 15 ml tube.

7. Spin down at $180 \times g$ for 5 min.

8. Resuspend the pellet with human ESCs medium supplemented with 10 μM Y27632 to the concentration at 5×10^5 cells/ml.

9. Remove the solution from matrigel-coated plate and add 100 μl fresh human ESCs medium with 10 μM Y27632 into the plate.

10. Add 100 μl of cell suspension into matrigel-coated plate pre-added with 100 μl of human ESCs medium.

11. Incubate at 37 °C under 5 % CO_2.

12. On the next day, remove human ESCs medium.

13. Rinse with PBS.

14. Change medium with fresh endoderm differentiation medium 1 supplemented with both Activin and B27 at day 0, 2, 4, 6 from the onset of differentiation.

15. Switch the medium to endoderm differentiation medium 2 supplemented with both Activin and B27 at day 8 from the onset of differentiation and culture cells for 2 days (Fig. 1) (*see* **Note 8**).
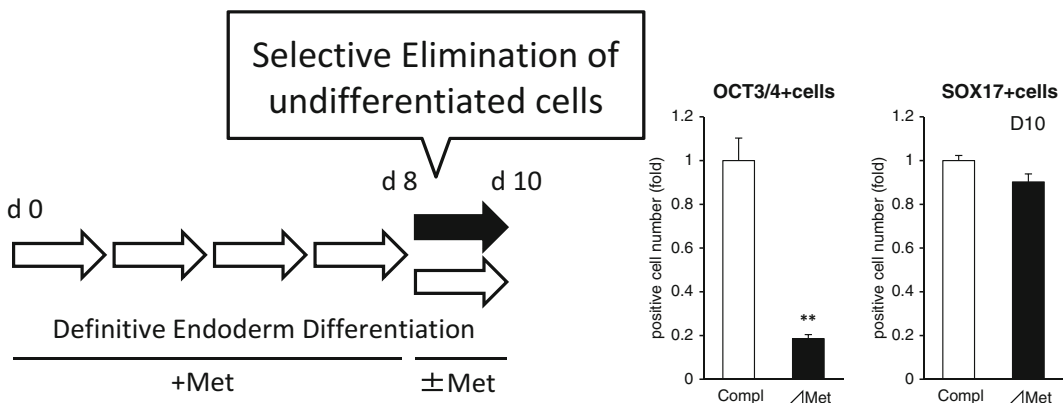
Fig. 1 Human ESCs (khES1) were induced into definitive endoderm through 10-day differentiation, with or without methionine deprivation from differentiation day (d) 8 to d 10. Methionine deprivation resulted in eliminating undifferentiated cells (marked by *OCT3/4* expression) without reducing endoderm cells (marked by *SOX17* expression). Error bars represent SEM ($n = 3$). Significant differences were determined by Student's *t* test; ** $p < 0.01$

4   Notes

1. Dissolve three tablets PBS (Sigma, P4417-100TAB) in 600 ml ultrapure water, autoclave, and store at room temperature.

2. Aliquot into 6 ml and store at −20 °C. Avoid freeze and thaw.

3. Dilute 2-mercaptoethanol (Sigma, M7522) to 0.1 M with PBS (i.e. 2-mercaptoethanol (Sigma, M7522) 100 μl/PBS 14.1 ml. Store at 4 °C and use within 1 month.)

4. Dissolve 0.2 g gelatin (Sigma, G9391) in 200 ml ultrapure water. Incubate at room temperature for 1 h, and autoclave, store at room temperature.

5. One day before plating, human ESCs are cultured in human ESCs medium supplemented with 10 μM Y27632. At 80 % confluence, human ESCs are plated.

6. Dissolve 5 mg Y27632 in 1.5 ml distilled water to make 10 mM stock solution. Aliquot into 50 μl and store at −80 °C.

7. Dilute 5 ml Matrigel with 5 ml DMEM (Invitrogen, 11995-075, high Glucose). Aliquot into 100 μl and store at −20 °C. Dilute ten times with DMEM before use.

8. You can combine this procedure with further differentiation (that is, hepatic and pancreatic differentiation, etc.) by continuing cell culture hereafter.

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Derivation of Endothelial Cells and Pericytes from Human Pluripotent Stem Cells

Sravanti Kusuma and Sharon Gerecht

Abstract

Blood vessels serve as the lifeline of nearly all living tissue. Vascular cells derived from human pluripotent stem cells hold great potential for clinical use in the regeneration of diseased vasculature and construction of blood vessels in engineered tissue. By deriving these cells in a controllable and clinically relevant manner harnessing physiological cues, we can obtain populations of cells amenable for transplantation. In this chapter, we describe methods to differentiate human pluripotent stem cells toward a bicellular population of early vascular cells using low oxygen cues, guide these subpopulations into mature endothelial cells and pericytes, and expand the vascular derivatives.

Keywords: Endothelial cells, Hydrogels, Pericytes, Pluripotent stem cells

1 Introduction

A major roadblock in regenerative medicine is the recreation of functional vasculature. The ability to rebuild blood vessels in the lab facilitates the progression of engineered tissues for transplantation as well as the development of innovative therapies to repair damaged tissue (1). Cell-based therapies have great utility toward these ends, offering permanent and effective strategies to rebuild vasculature. Because harvested somatic cells are difficult to expand in vitro and may damage the donor site, human pluripotent stem cells (hPSCs) hold greater potential for cell-based vascular reconstruction (2). Human PSCs are known for their ability to differentiate into every cell type and to have essentially limitless renewal capacity in vitro. Moreover, with the advent of a new subclass of hPSCs, human induced PSCs (hiPSCs), which are derived from somatic cells reprogrammed with key pluripotent factors, vascular reconstruction is now possible at a patient-specific level (3).

1.1 Endothelial Cells

Endothelial cells (ECs) form the inner lining of the entire vascular system. Cultured ECs are characterized by their cobblestone morphology, expression of endothelial-specific markers, such as vascular endothelial cadherin (VEcad), CD31, vascular endothelial growth

factor-2, and von Willebrand factor, and functionalities, such as lectin binding, capillary-like formation when cultured on Matrigel, and incorporation of DiI-labeled acetylated low density lipoprotein. ECs are intrinsically able to form vascular networks, but these structures often regress without the recruitment of a stromal cell type.

Furthermore, to rebuild larger vascular structures, a large number of cells must be available for transplantation (4). Toward this end, the ability for ECs to be passaged and expanded in the lab is an important property for EC derivatives to exhibit (5).

1.2 Pericytes

Pericytes are the predominant stromal cell type in microvasculature, but have slightly varying properties based on different vessel types. The expression of α-smooth muscle actin (αSMA) and the transmembrane chondroitin sulfate proteoglycan neuron-glial 2 (NG2) helps distinguish pericytes in different vessel types (6); pericytes of the capillaries are NG2$^+$αSMA$^-$, of the venules are NG2$^-$αSMA$^+$, and of the arterioles are NG2$^+$αSMA$^+$. When cultured, however, pericytes are positive for both of these markers. Other common markers used to characterize pericytes are calponin and PDGFRβ (7, 8).

1.3 Low Oxygen Tension as a Regulator of Vascular Differentiation

An important signal in vascular differentiation is oxygen tension, owing to the fact that ECs are in direct contact with the blood, positioning them as the first responders to changes in O_2 levels. A number of studies have implicated hypoxia, or low oxygen tension, in EC differentiation from hPSCs (9–12). Hypoxia is typically characterized as O_2 levels less than 5 % O_2, compared to an atmospheric oxygen level of 21 %. A recent study examining the effect of 1 % and 5 % O_2 differentiation conditions demonstrated that hypoxic conditions downregulate pluripotent markers while simultaneously increasing angiogenic and vasculogenic genes in hESCs (9). Moreover, just 24 h of 5 % O_2 differentiation yielded a population exhibiting 50 % CD34 positive expression. When these derived ECs were transplanted into an infarct, they were able to alleviate fibrous scar tissue by 50 %.

Deriving vascular cells from hPSCs must be done in a manner that is amenable to clinical translation. Specifically, derivation should occur in a controllable manner and in feeder-free conditions. In this chapter, we present a clinically relevant protocol harnessing low oxygen cues to differentiate hPSCs to early vascular cells (EVCs), a bicellular population made up of early ECs and pericytes, that can be further differentiated toward mature cell types. Finally, we conclude with assays to demonstrate the phenotype of our derivatives.

2 Materials and Equipment

Prepare and store all medium at 4 °C. Medium should be warmed to 37 °C prior to use.

2.1 *Equipment*	1. UV Lamp.
	2. Biosafety cabinet.
	3. Fume hood.
	4. Centrifuge.
	5. FACS machine.
	6. Light microscope.
	7. Incubator.
	8. Hermetically sealed hypoxia chamber.
	9. 5 % O_2 tanks.

2.1 Equipment

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2.2 EVC Differentiation

1. Culture of undifferentiated hPSCs to ~80 % confluence of colonies, on MEF feeder layers or feeder-free conditions in six-well culture dishes.
2. Phosphate buffer saline (PBS).
3. 15 ml and 50 ml sterile conical tubes.
4. 5 ml and 10 ml sterile serological pipettes.
5. 10 and 1,000 µl micropipette tips and pipettors.
6. EDTA: 5 mM EDTA in PBS, supplemented with 1 % (v/v) Hyclone FBS and 0.1 % (v/v) β-mercaptoethanol.
7. 40 µm strainer.
8. Hemocytometer.
9. Collagen type IV-coated six-well plates.
10. Early differentiation medium: alpha Minimum Essential Medium supplemented with 10 % Hyclone FBS and 0.1 mM β-mercaptoethanol.
11. Millipore Stericup Vacuum Filter 500 ml.
12. TrypLE™.
13. EVC differentiation medium: Endothelial Basal Growth Medium supplemented with 2 % serum (PromoCell), 50 ng/ml vascular endothelial growth factor (VEGF), 10 µM TGFβ inhibitor, SB431542 (Tocris), and 0.1 % penicillin-streptomycin antibiotic.

2.3 EC Maturation

1. MACS buffer: 0.5 % bovine serum albumin (BSA) and 2 mM EDTA in PBS.
2. Conjugated antibody: PE Mouse Anti-Human VEcad (BD).
3. Anti-PE microbeads (Miltenyi Biotec).
4. MACS MS column (Miltenyi Biotec).

2.4 Pericyte Maturation	Pericyte maturation medium: DMEM (without sodium pyruvate) supplemented with 10 % heat-inactivated FBS.

2.5 Characterization of Derived Vascular Cells

1. FACS tubes.
2. FACS buffer: 0.1 % BSA in PBS.
3. 1.5 ml Eppendorf tubes.
4. Conjugated antibody: PE Mouse Anti-Human VEcad (BD).
5. Conjugated antibody: PE Mouse Anti-Human CD31 (BD).
6. Conjugated antibody: PE Mouse Anti-Human Tra-1-60 (BD).

3   Methods

Carry out all procedures at room temperature and in a sterile laminar flow cabinet, unless otherwise specified.

3.1 EVC Derivation Using Low Oxygen Conditions

1. Early differentiation medium: mix into a 500 ml Stericup: 450 ml alpha Minimum Essential Medium, 50 ml Hyclone FBS, and 0.1 mM β-mercaptoethanol. Filter and store at 4 °C. (Can be used up to 1 month after preparation.)

2. Treat undifferentiated hPSC colonies grown in six-well plates with 0.5 ml EDTA per well (*see* **Note 1**).

3. Incubate at 37 °C for 10–15 min.

4. Add 1 ml early differentiation medium per well. Gently scratch the well's surface with a serological pipette to ensure complete cellular detachment.

5. Collect cell suspension in sterile 15 ml conical tube.

6. Centrifuge for 3 min at 240 × *g* and 4 °C.

7. Aspirate supernatant and resuspend pellet in early differentiation medium. Repeatedly pipette the cell suspension up and down using a 1,000 μm micropipette tip to obtain a single-cell suspension.

8. Filter the cell suspension through a 40 μm strainer to remove aggregates.

9. Count cells and calculate required number of wells to culture cells at a density of 500,000 cells per well.

10. Dilute single-cell suspension such that it is at a final volume of 1 ml per well.

11. Add 5 ml early differentiation medium per well on a collagen type IV-coated six-well plate.

12. Add 1 ml of cell suspension per well onto collagen IV-coated dishes. This day of seeding is designated "day 0."

13. Add a few drops of sterile water to a petri dish and place in the bottom of hypoxia chamber.

14. Sterilize chamber under UV for at least 15 min.

15. Add cell culture plates to chamber and seal.

16. Flush chamber with 5 % O_2 three times for 3 min, with each flush 30 min apart.

17. Culture in 37 °C incubator.

18. After 3 days, aspirate medium and replace with 6 ml fresh early differentiation medium.

19. Repeat steps 16 and 17.

20. On day 6, remove early differentiation medium and add 1 ml PBS per well.

21. Aspirate PBS.

22. Add 0.5 ml TrypLE per well.

23. Incubate in 37 °C incubator. After 5 min, ensure that cells have detached using a light microscope.

24. Add 1 ml early differentiation medium per well and collect cell suspension in a 15 ml sterile conical tube.

25. Centrifuge for 3 min at 240 × g and 4 °C.

26. Aspirate supernatant and resuspend pellet in EVC differentiation medium. Repeatedly pipette the cell suspension up and down using a 1,000 μm micropipette tip to obtain a single-cell suspension.

27. Filter cell suspension through a 40 μm strainer to remove aggregates.

28. Count cells and calculate required number of wells to culture cells at a density of 12,500 cells per cm^2.

29. Dilute cell suspension such that they are at a final volume of 1 ml per well.

30. Add 1 ml EVC differentiation medium per well on a collagen IV-coated dish.

31. Plate 1 ml of cell suspension per well onto collagen IV-coated dishes. Culture in 37 °C incubator at 5 % CO_2 (*see* **Notes 2** and **3**).

32. Change medium every other day.

33. On day 12, remove EVC differentiation medium and add 1 ml PBS per well.

34. Aspirate PBS.

35. Add 0.5 ml TrypLE per well.

36. Incubate in 37 °C incubator. After 5 min, ensure cells have detached using a light microscope.

37. Add 1 ml EVC differentiation medium per well and collect cell suspension in a sterile tube.

38. Centrifuge for 3 min at 240 × *g* and 4 °C.

39. Aspirate supernatant and resuspend pellet in appropriate medium.

40. Strain cell suspension through a 40 μm strainer to remove aggregates.

41. Count cells and proceed with desired downstream analysis, maturation toward ECs (Section 3.2), or maturation toward pericytes (Section 3.3).

42. EVCs derived under low oxygen conditions throughout the 12 day culture ("Continuous") or through the first 6 days of culture ("Primed") will exhibit a different morphology and greater expression of EC markers compared to EVCs derived under atmospheric conditions ("Control") (Fig. 1).

3.2 EC Maturation

1. Resuspend no more than 1×10^6 cells in 100 μl MACS buffer.

2. Add 10 μl of VEcad-PE antibody.

3. Vortex to mix.

4. Incubate on ice and in the dark for 30–45 min.

5. Centrifuge cells for 3 min at 240 × *g* and 4 °C.

6. Remove supernatant.

7. Resuspend in 1 ml MACS buffer and centrifuge for 3 min at 240 × *g* and 4 °C.

8. Remove supernatant.

9. Add 80 μl MACS buffer and 20 μl anti-PE microbeads.

10. Incubate for 15 min in the fridge in the dark.

11. Repeat steps 5–8.

12. Resuspend in 1 ml MACS buffer.

13. Place MS MACS column in magnetic column holder and a 15 ml conical tube to collect eluate.

14. Add 1 ml MACS buffer to column to wet.

15. After buffer has completely eluted, add cells through a 40 μm strainer.

16. Allow solution to completely pass through column and then add 0.5 ml MACS buffer to the column three times, adding only when the previous buffer and completely eluted.
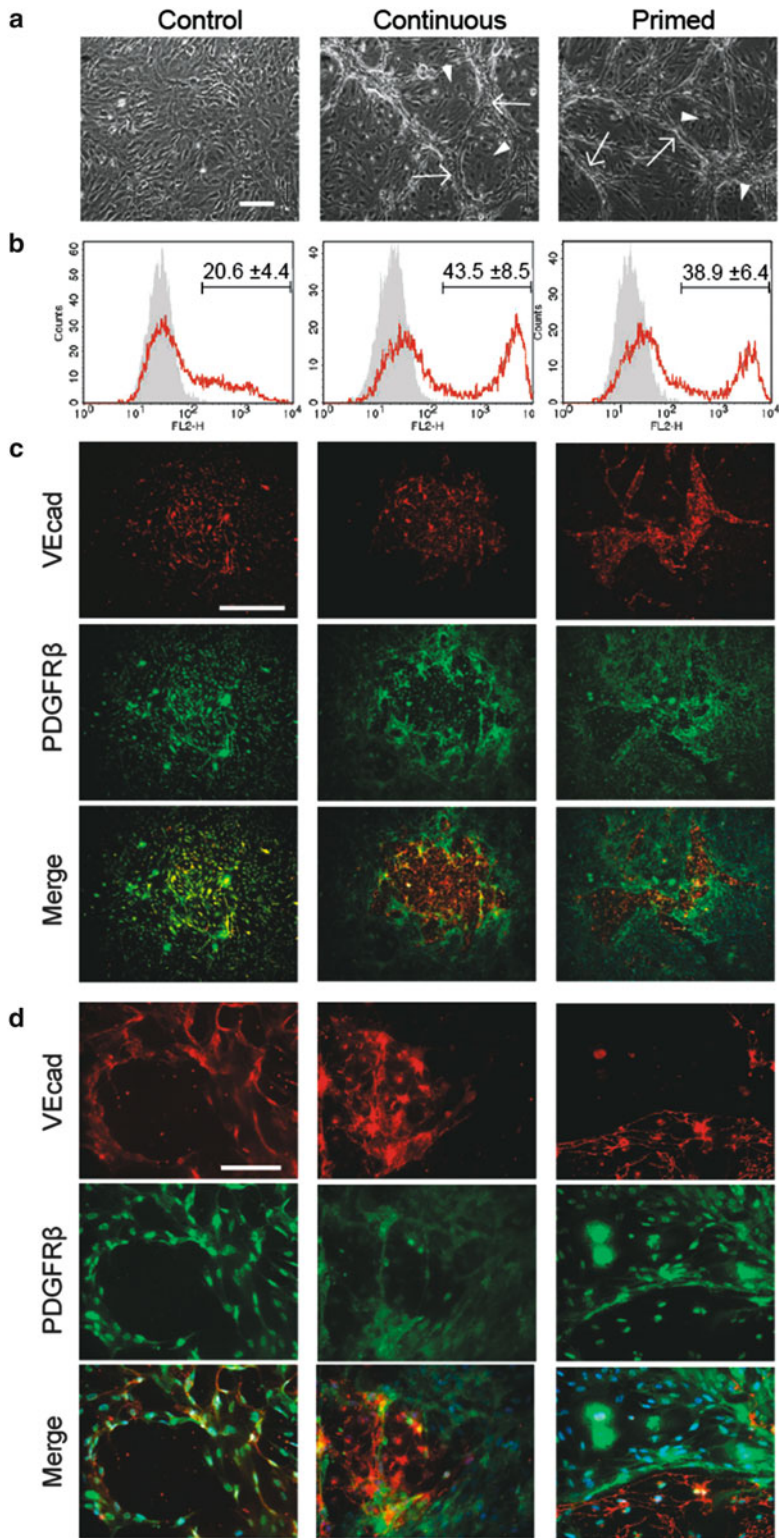
17. After three washes, remove column from magnet and place in a sterile 15 ml tube.

18. Add 1 ml MACS buffer and use plunger to forcefully remove cells in one motion (*see* **Note 4**).

19. Centrifuge cells for 3 min and $240 \times g$ and 4 °C.

20. Remove supernatant.

21. Add 1–2 ml EVC differentiation medium.

22. Repeat steps 19–21.

23. Replate cells on collagen IV-coated dishes at a cell density of 12,500 cells per cm^2.

24. Culture in 37 °C incubator at 5 % CO_2.

25. Change medium every other day.

26. Collect after 6 days for further analysis. Flow cytometry and immunofluorescence staining of these derivatives will reveal VEcad+CD31+CD146+ cells with membrane localization of VEcad and CD31, lectin binding, cytoplasmic expression of eNOS and von Willebrand factor (vWF), and uptake of acetylated low density lipoprotein (AcLDL).

3.3 Pericyte Maturation

1. Seed EVCs at a cell density of 12,500 per cm^2 onto tissue culture-treated wells in pericyte maturation medium.

2. Culture in 37 °C incubator at 5 % CO_2.

3. After 3 h, aspirate medium from wells and add fresh pericyte maturation medium.

4. Change medium every other day.

5. Collect after 6 days for further analysis. Flow cytometry and immunofluorescence staining of these derivatives will reveal enrichment in pericyte markers CD73, NG2, PDGFRβ, and CD44; depletion of EC markers VEcad and CD31; and appropriate localization of PDGFRβ, NG2 proteoglycan, and filamentous calponin expression.

3.4 Expansion of EC Derivatives

1. From Section 3.2, wash EC cultures with 1 ml PBS per well.

2. Add 0.5 ml TrypLE per well.

3. Incubate in 37 °C incubator. After 5 min, ensure cells have detached using a light microscope.

Fig. 1 EVC differentiation in varying low oxygen conditions. Comparison of control, continuous, and primed EVCs demonstrated by (**a**) light microscopy images (*arrows* indicate elongated cell bundles; *arrowheads* indicate cobblestone area-forming cells; scale bar is 100 μm), (**b**) flow cytometry for VEcad expression, and (**c**, **d**) immunofluorescence images of VEcad (*red*) and PDGFRβ (*green*). Nuclei in *blue*. Scale bar in **c** is 500 μm; scale bar in **d** is 100 μm. Adapted with permission from Lippincott Williams and Wilkins/Wolters Kluwer Health (12)

4. Add 1 ml EVC differentiation medium per well and collect cell suspension in a sterile tube.

5. Centrifuge for 3 min at $240 \times g$ and 4 °C.

6. Aspirate supernatant and resuspend pellet in EVC medium.

7. Culture cells at 15,000 cells per cm^2 on collagen IV-coated dishes.

8. Culture in 37 °C incubator, changing medium every other day.

9. Allow culture to grow to confluence (approximately 6–7 days) before passaging.

10. To calculate fold expansion, divide the total number of cells after each passage by the number of cells initially seeded at each passage.

3.5 Analysis by Fluorescence-Activated Cell Sorting (FACS)

1. Collect EVC, EC, or pericyte cell cultures separately with TrypLE as described above.

2. Centrifuge for 3 min and $240 \times g$ and 4 °C.

3. Resuspend pellet in FACS buffer, filter through a 40 μm strainer, and count cells.

4. Resuspend all cells to obtain a concentration of 200,000 cell per 100 μl FACS buffer.

5. Aliquot 100 μl of the cell suspension into 1.5 ml Eppendorf tubes.

6. Add appropriate antibody solution (such as VEcad-PE, CD31-PE, or Tra-1-60-PE, per the antibody manufacturer guidelines). For each antigen-specific staining, include an isotype control staining.

7. Vortex.

8. Incubate the samples on ice in the dark for 30–45 min.

9. Add 1 ml of FACS buffer to tubes and centrifuge for 3 min and $240 \times g$ and 4 °C.

10. Aspirate supernatant.

11. Repeat steps 9 and 10.

12. Resuspend pellet in 500 μl FACS buffer and strain through a 40 μm strainer to eliminate cell aggregates.

13. Take the tubes on ice to the FACS facility and run the FACS equipment (*see* **Note 5**).

4 Notes

1. Undifferentiated, healthy cultures of hPSCs are critical as starting populations. Cultures contaminated by differentiated cells will greatly affect differentiation potential.

2. Cell seeding density on day 6 is critical to ensure day 12 EVC phenotype. If too many cells are seeded, confluent cultures will inhibit VEcad expression.

3. Low oxygen culture is not essential for the second half of EVC differentiation. It can be used as a tool to modify the resulting EC to pericyte ratio in the EVC population.

4. If purity is not achieved on first MACS separation, it will be necessary to run another MACS separation with a fresh column on the sorted cells.

5. Samples may need to be strained a second time if clumping occurs.

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Differentiation of Human Embryonic Stem Cells on Periodontal Ligament Fibroblasts

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Abstract

Human embryonic stem cells' (hESCs) unlimited proliferative potential and differentiation capability to all somatic cell types makes them one of the potential cell sources in cell-based tissue engineering strategies as well as various experimental applications in fields such as developmental biology, pharmacokinetics, toxicology, and genetics. Periodontal tissue engineering is an approach to reconstitute the ectomesenchymally derived alveolar bone, periodontal ligament apparatus, and cementum tissues lost as a result of periodontal diseases. Cell-based therapies may offer potential advantage in overcoming the inherent limitations associated with contemporary regenerative procedures, such as dependency on defect type and size and the pool and capacity of progenitor cells resident in the wound area. Further elucidation of developmental mechanisms associated with tooth formation may also contribute to valuable knowledge based upon which the future therapies can be designed. Protocols for the differentiation of pluripotent hESCs into periodontal ligament fibroblastic cells (PDLF) as common progenitors for ligament, cementum, and alveolar bone tissue represent an initial step in developing hESC-based experimental and tissue engineering strategies. The present protocol describes methods associated with the guided differentiation of hESCs by the use of coculture with adult PDLFs and the resulting change of morphotype and phenotype of the pluripotent embryonic stem cells toward fibroblastic and osteoblastic lineages.

Keywords: Human embryonic stem cell, Periodontal ligament fibroblastic cell, Directed differentiation, Osteogenic induction, Cell coculture, Periodontal tissue engineering

1   Introduction

Human embryonic stem cells (hESCs) posses the ability to differentiate into all somatic and germ line representatives, and given their virtually unlimited proliferative potential, the cell type offers unique advantages in experimental elucidation of developmental mechanisms, testing the effects of newly developed drugs at the cellular level, revealing potential teratogenic and toxic effects of existing drugs on cellular differentiation, and also may provide unlimited cell source for prospective tissue engineering applications (1–3). These general utilities apply to a variety of tissues, including tooth supporting structures. Periodontal apparatus consists of three different but related tissues, namely, cementum on the tooth root surface, alveolar bone of the socket wall, and periodontal

Table 1
Fibrogenic differentiation of hESCs on hPDLFs for 3 weeks[a]

| | % Staining | | | | | | |
| | SSEA-4 | Col-I | Col-III | FBN | FSP | Vimentin | Pan-CK |
Weeks	Colony/SC (2-D)	Colony/SC (2-D)	Colony/SC (2-D)	Colony/SC (2-D)	Colony/SC (2-D)	Colony/SC (2-D)	Colony/SC (2-D)
1	18.2 ± 3.48/ 6.9 ± 1.31	12.3 ± 1.53/ 3.57 ± 1.40	NA/NA	7.67 ± 0.58/ 4.0 ± 1.28	4.33 ± 2.52/ 3.13 ± 0.80	2.33 ± 0.58/NA	3.77 ± 1.47/ NA
2	4.8 ± 1.85/NA	12.3 ± 2.08/ 5.27 ± 1.16	NA/11.1 ± 4.78	7.67 ± 2.08/ 9.0 ± 2.93	11.3 ± 3.51/ 6.43 ± 2.0	19.7 ± 7.77/ 16.3 ± 2.84	10.5 ± 4.52/ 14.4 ± 1.71
3	NA/NA	15.3 ± 1.15/ 15.1 ± 2.49	NA/21.8 ± 3.73	18.7 ± 2.52/ 20.2 ± 3.08	13.3 ± 3.79/ 9.27 ± 1.23	19.3 ± 4.51/ 16.5 ± 1.15	10.2 ± 2.44/ 23.7 ± 3.52

SC single cell, 2-D monolayer colony areas, *colony* three-dimensional colony areas, *SSEA-4* antistage-specific embryonic antigen-4, *Col-I* anticollagen type I, *Col-III* anticollagen type III, *FBN* antifibronectin, *FSP* antifibroblast surface protein, *Vimentin* antivimentin, *Pan-CK* antipan-cytokeratin, *NA* not available

[a]Immunohistomorphometric data representing mean ± standard deviation of staining percent for specific undifferentiation-related stem cell, mesenchymal, fibrogenic, and epithelial markers at indicated time points

ligament connecting them (4). All of these are descendants of ectomesenchyme, a mesenchymal tissue of neural crest origin, and progenitor cells in the periodontal ligament contribute to the regeneration of the lost structures to some extent with contemporary regenerative procedures (5). In order to achieve complete regeneration of periodontal defects with tissue engineering, the cells to be used should bear the potential to differentiate into cells of all three tissue types, like periodontal progenitors (6). Studying periodontal development and ectomesenchymal differentiation in its early phases with cells at initial stages of lineage development could substantially contribute to the understanding of tooth development as well as periodontal regeneration processes (7). Recent research indicate the possibility to induce human embryonic stem cells into odontogenic (8) and mesenchymal progenitors (9), providing the basis for establishing cell source in dental and periodontal tissue engineering strategies. Human embryonic stem cells may become a useful cell source with unlimited supply; however, the differentiation to periodontal progenitors is a prerequisite for their successful utilization. Overcoming immune rejection and demonstration of the efficacy and safety of the specifically designed tissue engineering structures are other challenges for using the cells in clinical therapies (10). Our group has established protocols for initial differentiation toward periodontal lineages using coculture with adult human periodontal ligament fibroblasts (hPDLFs) (*see* Tables 1 and 2) (11, 12). Here, the culture expansion of hPDLFs and human embryonic stem cells are described, followed by the coculture procedures and immunohistochemical and RT-PCR detection of differentiation marker expression as well as microscopic demonstration of morphological changes during the experiments (*see* Fig. 1). The methodology represents initial steps in obtaining definitive periodontal progenitors, which could be used in experimental and prospective clinical applications in the field of periodontology.

Table 2
Osteogenic differentiation of hESCs on hPDLFs for 4 weeks[a]

	% Staining				
Days/area	ALP	OSN	OSP	BSP	OSC
1/2-D	19.5 ± 4.05	NA	NA	NA	NA
28/2-D	9.07 ± 2.25	NA	3 ± 0.954	16 ± 2.51	8.3 ± 2.91
28/3-D	19.9 ± 2.36	7.87 ± 1.39	14.1 ± 2.11	25.1 ± 2.4	10 ± 2.26

2-D monolayer colony areas, *3-D* three-dimensional colony areas, *ALP* antialkaline phosphatase, *OSN* antiosteonectin, *OSP* antiosteopontin, *BSP* antibone sialoprotein, *OSC* antiosteocalcin, *NA* not available
[a]Immunohistomorphometric data representing mean ± standard deviation of staining percent for specific osteogenic differentiation markers at indicated time points
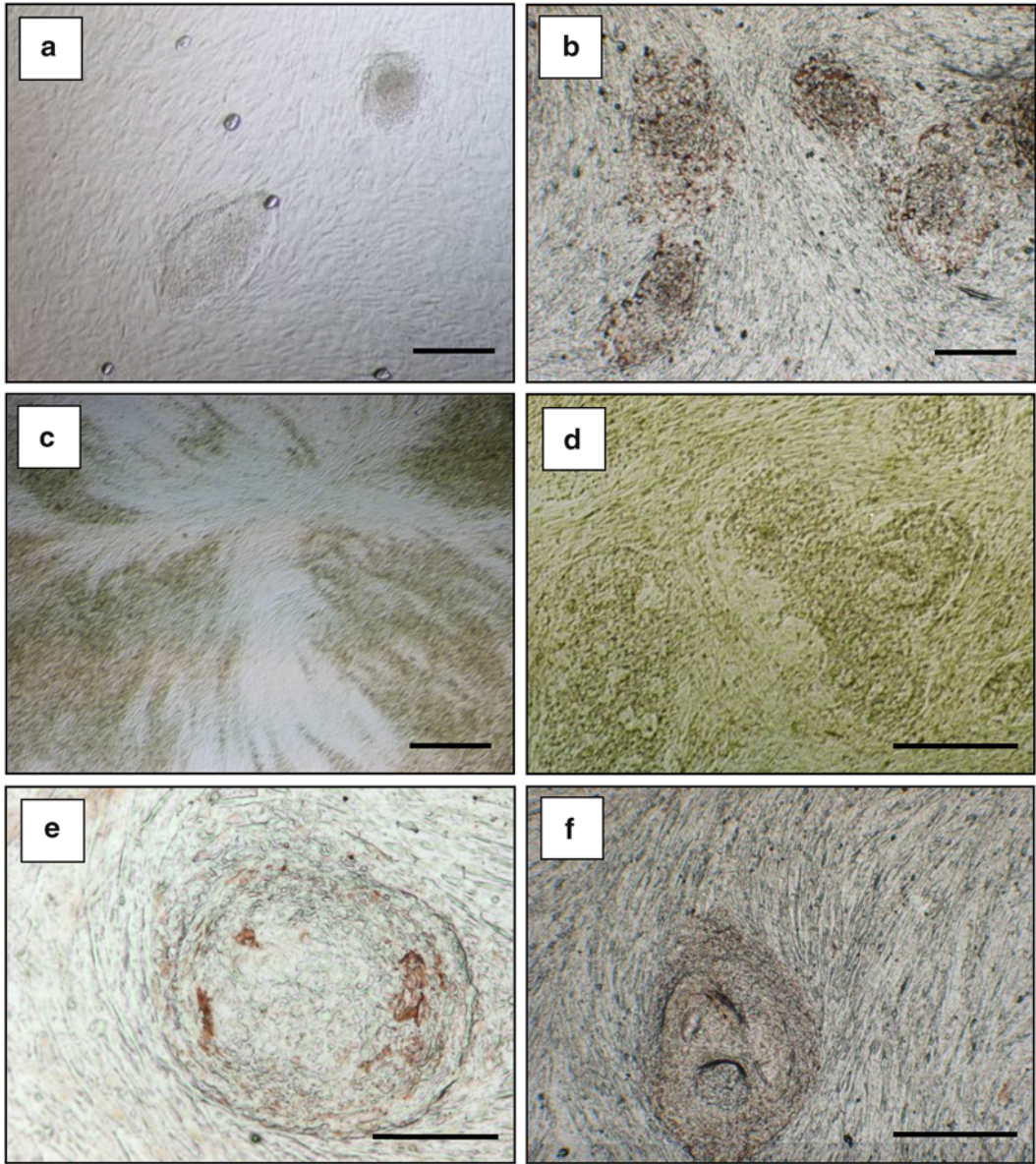
Fig. 1 Undifferentiated and differentiated human embryonic stem cells. (**a**) Undifferentiated hESC colonies on MEFs at day 5 after passage. (**b**) Colonies stain positively for SSEA-4. (**c**) After differentiation for 21 days in coculture with PDLFs, embryonic stem cells spread in 2-D and intermingle with adult fibroblastic cells. (**d**) Differentiation in osteogenic medium for 21 days results with more compact and rounded cell clusters having 3-D areas and rough surface appearance. (**e**) Round colonies differentiated under osteogenic induction conditions exhibit staining foci for BSP at day 28. (**f**) Slight staining for osteocalcin at day 28 indicates early phase of osteogenic differentiation. Scale bars = 200 μm

2   Materials

2.1   Culture of hPDLFs

1. Expansion medium (PDLF-EM) was prepared with Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Invitrogen, Paisley, UK, Cat. No. 12800-082) supplemented with: fetal bovine serum, 10–15 % (FBS; Sigma, St. Louis, MO, USA, Cat. No. F6178); nonessential amino acid stock solution, 1 % (NEAA; Gibco, Cat. No. 11140-035); L-glutamine, 1 mM (Gibco, Cat. No. 25030-032); and penicillin-streptomycin, 100 u/ml–100 μg/ml (Sigma, Cat. No. A5955).

2. Solution of trypsin, 0.05 %, and ethylenediaminetetraacetic acid, 0.53 mM (EDTA; Gibco, Cat. No. 25300054).

3. Mitomycin C (Sigma, Cat. No. M4287), dissolved in PBS at 1 mg/ml and stored in single-use aliquots of 100 μl at −20 °C.

4. Tissue culture flasks, 25 cm^2 and 75 cm^2 (Corning, Schiphol Rijk, The Netherlands, Cat. Nos. 430168 and 430641, respectively).

5. Centrifuge tubes, 50 ml (Corning, Cat. No. 430921).

2.2   Culture of hESCs

1. Human embryonic stem cell line (HUES-9, HUES Facility, Melton Laboratory, HHMI, Harvard University, Cambridge, MA, USA).

2. Expansion medium (ES-EM) was prepared with KnockOut™ Dulbecco's Modified Eagle's Medium (KO™-DMEM; Gibco, Cat. No. 10829-018), supplemented with: KO™-Serum Replacer, 20 % (KO™-SR, Gibco, Cat. No. 10828); nonessential amino acid stock solution, 1 % (NEAA; Gibco, Cat. No. 11140-035); L-glutamine, 1 mM (Gibco, Cat. No. 25030-032); penicillin-streptomycin, 100 u/ml–100 μg/ml (Sigma, Cat. No. A5955); and basic fibroblast growth factor, 8 ng/ml (bFGF, Sigma, Cat. No. F0291). bFGF is dissolved in KO-DMEM™ at 1 mg/ml concentration and stored in single-use aliquots at −40 °C.

3. Six-well culture plates (Corning, Cat. No. 3516).

4. Collagenase type IV, 1 mg/ml (Gibco, Cat. No. 17104-019).

2.3   hESC Transfection

1. Green fluorescent protein (GFP)-encoding plasmid pQBI PGK (Quantum Biotech Inc., Q-Biogene, Montreal, Canada, Cat. No. AFP2043).

2. FuGENE6 transfection reagent (Roche Diagnostics, Basel, Switzerland).

3. Vials, DNase-free, 0.65 ml (Corning, Cat. No. 3208).

4. G418 (Sigma, Cat. No. A1720).

2.4 Fibrogenic Differentiation	1. Thermanox™ coverslips, tissue culture treated, 13 and 25 mm (Nalge Nunc Int., Rochester, NY, USA, Cat. Nos. 174950 and 174985).
	2. Six-well culture plates (Corning, Cat. No. 3516), 15-ml centrifuge tubes (Corning, Cat. No. 430766), and micropipette tips 1 ml (Corning, Cat. No. 4809).
	3. Differentiation medium (ES-DM): same as ES-EM except that KO™-SR was replaced with 20 % FCS and no bFGF added.
	4. Soybean trypsin inhibitor, 1 mg/ml (Gibco, Cat. No. 17075-029).

2.5 Osteogenic Induction

1. Osteogenic induction (ES-OI) medium was prepared by supplementing ES-DM medium with: β-glycerophosphate, 10 mM (Sigma, Cat. No. G9891); ascorbic acid, 50 μg/ml (Sigma, Cat. No. A-4544); and dexamethasone, 10^{-7} M (Sigma, Cat. No. D4902).

2.6 Immunohisto-chemistry

1. Methanol (Riedel-de Haën, Fluka, Buchs, Switzerland, Cat. No. 24229).

2. Permeabilization solution: Triton X, 0.01 % (v/v) (Sigma, Cat. No. T8787).

3. Endogenous hydrogen peroxidase block (Lab Vision, Fremont, CA, USA, Cat. No. TA-125-HP).

4. Nonspecific binding block (Lab Vision, Cat. No. TA-125-UB).

5. Antibody dilution buffer: bovine serum albumin, 2 % (w/v) (BSA; Sigma, Cat. No. A4919) in PBS.

6. Primary antibodies:

    (a) AntiSSEA-4, goat polyclonal IgG (Santa Cruz Biotech., Santa Cruz, CA, USA, Cat. No. SC-21704).

    (b) Antifibroblast surface protein, mouse monoclonal IgM (Sigma, Cat. No. F4771).

    (c) Antifibronectin, mouse monoclonal IgM (Sigma, Cat. No. F6140).

    (d) Anticollagen type I, goat polyclonal IgG (Santa Cruz Biotech.; Cat. No. SC-8786).

    (e) Anticollagen type III, mouse monoclonal IgG1 (Sigma, Cat. No. C7805).

    (f) Antivimentin, mouse monoclonal IgM (Sigma, Cat. No. V6630).

    (g) Antipan-cytokeratin, mouse monoclonal IgG1 (Santa Cruz Biotech.; Cat. No. SC8018).

    (h) Antiosteonectin, mouse monoclonal IgG (Alexis, San Diego, CA, USA; Cat. No. 804-317-C100).

(i) Antiosteopontin, goat polyclonal IgG (Santa Cruz Biotech.; Cat. No. SC-21742).

(j) Antibone sialoprotein, rabbit polyclonal IgG (Alexis, Cat. No. 210-312-R100).

(k) Antiosteocalcin, goat polyclonal IgG (Santa Cruz Biotech.; Cat. No. SC-18319).

7. Secondary Antibodies: biotinylated goat anti-mouse IgM (Cat. No. 62-6840), goat anti-mouse IgG (Cat. No. 81-6511), mouse anti-goat IgG (Cat. No. 81-6540), and goat anti-rabbit IgG (Cat. No. 65-6140), all from Zymed, San Francisco, CA, USA.

8. Detection system: Horseradish peroxidase enzyme system and AEC chromogen (Lab Vision, Cat. No. TA-125-HA).

2.7 SEM

1. Fixing solution: glutaraldehyde, 2.5 % (v/v) (Sigma-Aldrich, Cat. No. G7651) in 0.1 M sodium cacodylate buffer (Sigma, Cat. No. C0250).

2. Ethanol (Riedel-de Haën, Cat. No. 32221).

2.8 RT-PCR

1. mRNA extraction: Direct mRNA minipreparation kit (GenElute™, Sigma, Cat. No. DMN-70).

2. cDNA synthesis (SuperScript™ first-strand synthesis system, Invitrogen, Cat. No. 11904-018).

3. Reverse transcription mixture: $MgCl_2$ (25 mM); DTT (0.1 M); random hexamer (50 ng/ml); dATP, dCTP, dGTP, and dTTP (10 mM each); reverse transcriptase (50 U/ml, SuperScript™ II); and RT buffer (10×), all from Invitrogen.

4. PCR mixture: *Taq* polymerase (5 U/μl), dNTP mixture (10 mM each), specific primers (10 pM each), $MgCl_2$ (50 mM), cDNA, dH_2O, and PCR buffer (10×), all from Invitrogen.

5. Gel: Agarose (Sigma, Cat. No. A5093).

6. Other chemicals: Trizma R base (Cat. No. T-1503), boric acid (Cat. No. B-6768), EDTA (Cat. No. ED2SS), all from Sigma.

3 Methods

3.1 Culture of hPDLFs

1. Scrape the periodontal ligament tissue from the middle third of a healthy tooth root (extracted for orthodontic treatment) with sterile surgical blades.

2. Transfer the explants into a 15-ml centrifuge tube in PDLF-EM containing 10× antibiotics.

3. Discard the medium, and wash the explants three times with sterile PBS at room temperature in a laminar air flow cabinet.

4. After final wash, add 2 ml of PDLF-EM into the tube and pipette back the explants, and then transfer them to a 25-cm^2 flask containing 8 ml PDLF-EM and place inside an incubator at 37 °C, 5 % CO_2.

5. Change the medium every 2–3 days, removing 4.0 ml and adding 4.0 ml of newly prepared PDLF-EM.

6. Continue to culture for 3–6 weeks until proliferating fibroblasts from explants become confluent on the flask surface.

7. Discard the medium, wash the cells three times with sterile PBS, add dropwise trypsin/EDTA at room temperature until just covering the surface, and keep the flask for 5 min inside the incubator.

8. Remove the flask, tap rigorously with hand to completely dissociate the rounded fibroblastic cells, and then add 7.0 ml of PDLF-EM.

9. Pipette out the cell suspension and transfer to a 15-ml centrifuge tube, wash the remaining cells (*see* **Note 1**) with additional 5.0 ml medium, and add to the tube.

10. Centrifuge the cell suspension for 5 min at 800 rpm, 25 °C.

11. Discard the supernatant, and add 3.0 ml of PDLF-EM to the remaining cell pellet.

12. Gently pipette up and down to resuspend the cells, then transfer the suspension into a 75-cm^2 flask containing 20.0 ml PDLF-EM, and designate as passage 1 (P-1).

13. Continue to culture for 2–3 days until cells reach confluence.

14. Change the medium 1 day before the next passage.

15. Continue the expansion by splitting cells 1:2 or 1:3, until obtaining desired cell number for the experiments.

3.2 Culture of hESCs

1. Remove the vial containing frozen cell line from the liquid nitrogen tank and quickly immerse in water at 37 °C.

2. Thaw the cells with gentle shaking until little ice crystal remains, then transfer the vial to the laminar air flow cabinet.

3. Pipette the cell suspension with a micropipette, and add dropwise (*see* **Note 2**) into a 15-ml centrifuge tube containing 12.0 ml ES-EM at 25 °C.

4. Centrifuge the cell suspension for 5 min, at 650 rpm, 25 °C.

5. Remove the supernatant (and DMSO-containing medium), and add 3.0 ml of ES-EM.

6. Pipette up and down very gently several times to barely dissociate cell clumps from one another.

7. Transfer the cells on six-well plates covered with mitotically inactivated primary mouse embryonic fibroblast (MEF) feeder cells.

8. Add ES-EM until reaching 3.0 ml/well, and then place the plate inside the incubator at 37 °C, 5 % CO_2.

9. Change the medium daily removing 2.5 ml and adding 2.5 ml.

10. Maintain the culture for 8–10 days, until ESC colonies with undifferentiated morphology become prevalent.

11. Select the colonies demonstrating the undifferentiated morphology under inverted microscope (*see* **Note 3**), and pick them up mechanically with a micropipette tip (*see* **Note 4**).

12. Colonies are dissociated into several particles during mechanical selection; transfer the clumps into ES-EM-containing 35-mm dish or 15-ml centrifuge tube.

13. Pipette the collected hES cell clumps several times, only to dissociate from one another (*see* **Note 5**), and then transfer the cells to six-well tissue culture plates with newly prepared MEFs.

14. Add ES-EM to reach 3.0 ml/well and shake the plate to ensure even distribution of hES cells before placing in the incubator.

15. Designate as Pn + 1 where "n" is the passage number at which line is received.

16. Change medium daily during expansion, and perform the next passage after 5 days, again selecting the colonies with undifferentiated morphology.

3.3 Transfection of hESCs

1. Remove the hESC-containing six-well culture plate at the third day following passage from the incubator and discard the medium.

2. Wash twice with KO™-DMEM, then add 400 μl of KO™-DMEM or ES-EM (*see* **Note 6**), and keep for 15 min in the incubator.

3. Add 97 μl of KO™-DMEM in sterile DNase-free 0.6-ml vial, then add 6 μl of FuGENE, and stir gently to mix the components for 5 min at room temperature.

4. Add 1 μg of plasmid DNA in a 20 μl of carrier to the mixture and mix; then keep for 20 min at room temperature.

5. Add 20 μl from the mix to every well containing 400 μl of KO™-DMEM or ES-EM, and then keep the plate in the incubator for 6 h.

6. Complement the medium to 3.0 ml/well adding ES-EM.

7. Change the whole medium after 24 h.

8. Observe the cells under fluorescent microscope at 24, 48, and 72 h post-transfection.

9. To determine the transfection percent, count the GFP + colonies under fluorescent microscope and all colonies under inverted light microscope.

10. To achieve stable transfection, select GFP + colonies, pick them up with a micropipette tip, and transfer to newly prepared MEFs.

11. Culture in the presence of 600 µg/ml G-418 for 14 days, with passages every other day in nonresistant MEFs, and every 5 days when gentamicin-resistant MEFs are used.

12. Select the GFP + colonies to be used for experiments.

3.4 Differentiation of hESCs

1. Seed PDLFs at a density of ~50,000 cells/cm^2 on Thermanox™ coverslips in six-well plates.

2. Incubate the cells in PDLF-EM until reaching confluence (2–3 days).

3. Mitotically inactivate the PDLFs through incubation with PDLF-EM containing 10 µg/ml mitomycin C for 2–3 h (*see* **Note 7**).

4. Wash several times with PDLF-EM to completely eliminate the antibiotic, and then incubate for 24 h in PDLF-EM.

5. Remove the hESC-containing plate from incubator at day 5 after the last passage and mechanically select the colonies with undifferentiated morphology.

6. Remove the colony particles with micropipette tip under inverted microscope in laminar air flow cabinet, and transfer the clumps into a 15-ml centrifuge tube containing 3.0 ml ES-EM.

7. Collect the hES cells from the same passage in the tube, and then pipette gently to dissociate the clumps.

8. Alternatively, add 2.0 ml of trypsin/EDTA, incubate for 5 min, and then dissociate the cells into single-cell suspension by gentle pipetting with a micropipette.

9. Quench the trypsin activity by adding soybean trypsin inhibitor.

10. Transfer the hESC colony particles (or single-cell suspension) to six-well plates on Thermanox™ coverslips covered with confluent layer of PDLF cells.

11. Add ES-EM until reaching 3.0 ml/well.

12. Place the cells inside the incubator at 37 °C, 5 % CO_2.

13. After 24 h, the hESC cell clumps will be largely attached to the PDLF surface (designate as day 1).

14. Discard the ES-EM medium and add the same amount of ES-DM or ES-OI medium for fibrogenic or osteogenic differentiation, respectively.

15. Daily change the medium during 28 days of differentiation experiments.

3.5 Immunohisto-chemistry

1. Remove the selected culture plates at predetermined time points from the incubator, discard the medium, and wash three times with PBS at room temperature.

2. Add 1.0 ml/well of ice-cold methanol, keep for 45 min at room temperature, and then discard the excess methanol and leave the plate to air-dry.

3. Permeabilize the fixed cells with 0.01 % (v/v) Triton X for 30 min, and wash three times with PBS.

4. Quench the endogenous peroxidase activity by incubating at room temperature with peroxide block for 10 min, and wash three times with PBS.

5. Block the nonspecific binding by incubating at room temperature with blocking solution for 10 min, and wash three times with PBS.

6. Incubate with primary antibody at predetermined concentration (by titration) for 1 h at room temperature or overnight at 4 °C.

7. Incubate with species and isotype-specific secondary antibody at predetermined concentration (by titration) for 30 min at room temperature, and wash three times with PBS.

8. Incubate for 20 min with avidin-biotin horseradish peroxidase enzyme system, and wash three times with PBS.

9. Incubate for 5–15 min at room temperature with AEC chromogen until red staining develops.

10. Wash three times with distilled water, mount, and visualize under the microscope.

3.6 SEM

1. Remove the selected culture plates (coverslips) at predetermined time points from the incubator, discard the medium, and wash three times with PBS at room temperature.

2. Fix with 2.5 % (v/v) glutaraldehyde in cacodylate buffer for 30 min at room temperature or overnight at 4 °C.

3. Dehydrate with graded ethanol series (50, 70, 95, and 100 %).

4. Dry in air.

5. Place samples on stubs.

6. Sputter-coat with gold and visualize under scanning electron microscope.

3.7 RT-PCR

1. Prepare cell culture for mRNA isolation. Put the cell suspension in a sterile 1.5-ml microfuge tube.

2. Centrifuge the tubes at maximum speed for 2 min at room temperature in a microfuge.

3. Discard all but 25 µl of each supernatant.

4. Use the direct mRNA miniprep kit's procedure.

5. Keep the mRNA in aqueous solution on ice.

6. Add the mRNA to reverse transcription mixture in 0.65-ml tubes and follow the procedure.

7. Amplify cDNA in aqueous solution. Transfer the cDNA to PCR tubes, and then add the PCR mixture. End volume of the mix solution is 50 µl (*see* **Note 8**).

8. Preheat the lid to 105 °C. Amplify the nucleic acids using denaturation (1 min at 95 °C), annealing (1 min at 55–60 °C), and polymerization cycles (1 min at 72 °C).

9. Amplify for 25–35 cycles.

10. Take samples (10 µl each) from the test reaction mixture and the four control reactions, run them by agarose electrophoresis, and stain the gel with ethidium bromide. Analyze the gel using UV visualization and documentation system.

4 Notes

1. Multiple explant particles may remain undissociated on the flask surface at this first passage, and further PDL fibroblasts can be expanded by continuing their culture.

2. Adding thawed cell suspension dropwise into freshly prepared media aims at reducing the osmotic shock that affects cell survival rate and is a general cell culture thawing rule.

3. The microscope should be located inside the laminar air flow cabinet, and the culture plate should be kept on a metal plate at 37 °C, minimizing temperature fluctuations which affect differentiation of hESCs.

4. The hESC colonies could be collected after enzymatic incubation with collagenase type IV for 5 min; however, this diminishes the selection ability of undifferentiated colonies, albeit shortens the time needed for passaging procedure.

5. Rigorous pipetting may result with increased dissociation into single cells, which subsequently differentiate spontaneously during expansion culture.

6. KO™-SR did not adversely affect the transfection efficiency in our experiments; therefore, ES-EM could be used instead of KO™-DMEM in the first 6 h of transfection.

7. The PDLFs may or may not be mitotically inactivated, for they proliferate slower than MEFs and contact inhibition at confluence further diminishes their expansion.

8. Mineral oil should be used to cover the PCR mixture.

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Derivation of Epithelial Cells from Human Embryonic Stem Cells as an In Vitro Model of Vocal Mucosa

Vlasta Lungova, Ciara Leydon, and Susan Thibeault

Abstract

Vocal fold epithelial cells are very difficult to study as the vocal fold epithelial cell lines do not exist and they cannot be removed from the healthy larynx without engendering a significant and unacceptable risk to vocal fold function. Here, we describe the procedure to create an engineered vocal fold tissue construct consisting of the scaffold composed of the collagen 1 gel seeded with human fibroblasts and simple epithelial progenitors seeded on the scaffold and cultivated at air–liquid interface for 19–21 days to derive the stratified squamous epithelium. This model of vocal fold mucosa is very similar in morphology, gene expression, and phenotypic characteristics to native vocal fold epithelial cells and the underlying lamina propria and, therefore, offers a promising approach to studying vocal fold biology and biomechanics in health and disease.

Keywords: 3D tissue constructs, Vocal fold epithelium, Differentiation of human embryonic stem cells, Air–liquid interface, Simple epithelial progenitors, Stratified squamous epithelium

1 Introduction

In vitro model of vocal fold mucosa represents a valid, reproducible 3D model of human origin (1). It has been developed to better understand the pathophysiology of diseases in the vocal fold, which can lead to improved treatment outcomes. Systematic in vivo study of vocal fold disorders is challenging as the vocal folds are housed deep within the larynx and are vulnerable to manipulation. Moreover, vocal fold epithelial cell lines do not exist nor they can be removed from the healthy larynx without engendering a significant and unacceptable risk to vocal fold function. The stem cell derived in vitro model represents a valid, reproducible model of vocal fold mucosa composed of collagen 1 gel seeded with human fibroblasts and populated by a continuous, large-scale supply of stable epithelial cells that demonstrate the key morphologic, genotypic, and phenotypic similarities of native vocal fold epithelial cells. This model serves as an invaluable tool for examining and manipulating cell signal pathways in development, health, and injury, modeling vocal fold damage and disease, and/or testing safety and effectiveness of novel cytoprotective and regenerative treatments in vitro.

2   Materials

2.1   Cell Lines

1. Human embryonic stem cell (hESC) line (WA09; passage 20–26) for derivation of simple epithelial progenitors (WiCell, UW Madison, WI).

2. Human fibroblasts T21 cell line (passage 5–6) for gel–cell constructs. This line was developed from pathogen-free vocal folds obtained from a 21-year-old male donor and has been characterized previously (2). The protocol was approved by the Institutional Review Board of the University of Wisconsin–Madison.

2.2   Culture Medium Preparation Procedure

Preparing culture medium for maintenance and differentiation of hESC must be done under sterile conditions at room temperature. Diligently follow all waste disposal regulations when disposing waste materials. Recommended storage for medium is 4 °C:

1. mTESR1 medium for hESC maintenance (STEMCELL Technologies, Vancouver, CA). mTeSR1 medium remains fresh for 14 days.

2. Unconditioned hESC medium (UM) supplemented with retinoic acid (RA) for derivation of simple epithelial progenitors:

   Mix the DMEM/F12 medium with 20 % knockout serum replacer (Life Technologies Corporation, Carlsbad, CA), 1x MEM nonessential amino acids, 1 mM L-glutamine, 0.1 mM 2-mercaptoethanol, and 1 mM all-trans retinoic acid (Sigma-Aldrich, St. Louis, MI). Prepare fresh UM + RA medium prior to differentiation of epithelial progenitors.

3. Flavin adenine dinucleotide (FAD) medium for differentiation and stratification of epithelial progenitors:

   Mix Ham's F-12/DMEM (3:1 ratio) with FBS (2.5 %), hydrocortisone (0.4 mg/ml), cholera toxin (8.4 ng/ml), insulin (5 mg/ml), adenine (24 mg/ml), EGF (10 ng/ml), penicillin (100 U/ml), and streptomycin (0.01 mg/ml). FAD medium should be prepared fresh every week.

4. 10 % DMEM medium for gel–cell mix preparation, adjust pH with 10 % DMEM with 1 N NaOH, required pH = 7.2–3.

2.3   Collagen 1 Gel Preparation Procedure

It is recommended that the collagen and other working solutions be chilled and kept on ice during the preparation of the collagen gel. Preparation of the collagen gel must be done under sterile conditions in a biological safety cabinet.

1. Place the following reagents on ice: Type I collagen (BD Biosciences, high concentration gel), sterile PBS (10×), sterile Cell Culture Water (dH$_2$O), sterile 1 N NaOH. The actual concentration of collagen 1 is printed on the product label and

certificate of analysis for each specific lot. Required final working concentration of collagen is 4 mg/ml.

2. Determine the final volume of collagen 1 and other reagents and adjust pH according to the following the manufacturer's calculations:

(a) Volume of Collagen needed

$$= \frac{(\text{Final Concentration}) \times (\text{Total Volume})}{(\text{Initial Concentration of Collagen})}.$$

(b) Volume of $10 \times$ PBS needed $= \dfrac{\text{Total Volume}}{10}$.

(c) Volume of 1 N NaOH needed $=$ Volume of Collagen
$$\times\, 0.017 \text{ ml}.$$

(d) Volume of dH_2O needed $=$ Total Volume
$$-\,(\text{Sum of } a + b + c).$$

3. Mix the volumes calculated for $10\times$ PBS, sterile NaOH, and sterile dH_2O in a sterile tube in a biological safety cabinet.

4. Add collagen to the tube with the reagents and pipet up and down to mix. Vortexing is not recommended.

3   Methods

3.1   Thaw and Maintain hESC

1. Aliquot and coat a six-well plate with the Matrigel as has been described previously (3), see **Note 1**.

2. Let the Matrigel sit for at least 1 h in a biological safety cabinet at room temperature to effectively coat the wells.

3. Remove the excess Matrigel and add 1.5 ml of culture medium (mTeSR1) into each well.

4. Thaw 1 vial of hESC (cell line WA09; passage 20–26) as has been described previously (3), to obtain 0.5 ml of the cell suspension (the cells mixed with 0.5 ml of mTeSR1 medium), see **Note 2**.

5. Plate the cells into the Matrigel coated plates. Slowly add 0.5 ml of cell suspension drop-wise into one well of the Matrigel coated six-well plate which already contains 1.5 ml mTeSTR1 medium. Final plating volume will be 2 ml/well.

6. Place the plate gently into the incubator, gently shake the plate front to back, pause, then, side to side and allow cells to attach overnight.

7. Maintain cells in mTeSR1 for their expansion. Feed the cells every day with 2 ml of mTeSR1 medium/well in a six-well

plate. It is acceptable to "double feed" once in a 7 day period. To double feed, add 4 ml of mTeSR1 medium per well on day one. The cells will not be fed on day two, and will resume normal feeding with 2 ml per well on day three.

8. Passage cells using Dispase or Versene, as has been described previously (3) prior you initiate differentiation experiments to allow cells to recover from thawing. Cells cultured in mTeSR medium are typically split 1:4 or 1:5 within 5–8 days. A thorough microscopic examination is required to determine the split ratio, *see* **Note 3**.

3.2 Differentiation of Simple Epithelial Progenitors

1. Prepare unconditioned hESC medium (UM) supplemented with retinoic acid (RA) for derivation of simple epithelial progenitors according to the Section 2.2.

2. Aspire off mTeSR medium and add UM + RA medium 2 ml per well in a six-well plate. Cultivate cells in UM + RA for 7 days with the medium changed daily to differentiate simple epithelial progenitors (4).

3. hES derived simple epithelial progenitors should be tested for pluripotency and simple versus stratified epithelial markers using standard molecular, histological, and/or immunohistochemical analyses.

3.3 Create a 3D Tissue Construct

1. Prepare scaffold that consists of collagen 1 gel seeded with human fibroblasts (T21 cell line, passage 5–6) (5). The scaffold serves to mimic the collagen-rich vocal fold lamina propria.

   (a) Prepare collagen 1 gel, *see* Section 2.3. Required final working concentration of collagen is 4 mg/ml.

   (b) Mix collagen 1 gel (80 % of final volume), $10\times$ DMEM (10 % final volume), pH 7.2–3, ice-cold FBS (10 % final volume) with 5×10^5 fibroblasts per ml.

   (c) Add 2 ml of gel–cell mix to each six-well insert and put in an incubator for 1 h.

   (d) Add 1 ml pre-warmed FAD (*see* Section 2.2) to each gel–cell mix and well.

   (e) Gently detach gel from walls with Pasteur pipet and let contract for 1 or 2 days.

2. Seeding of simple epithelial progenitors on the inserts:

   (a) After 1 or 2 days seed $2–3 \times 10^5$ simple epithelial progenitors in 100 µl of FAD on each insert and allow them to attach to the gel for 2 h in an incubator.

   (b) Add 1 ml FAD media on gel and 2 ml of media around insert.

   (c) Rinse with FAD and return to an incubator.
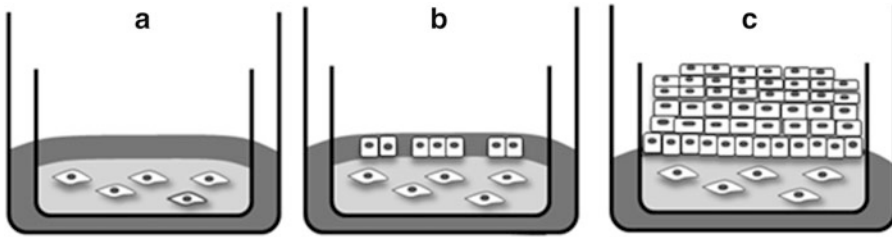
Fig. 1 Schematic of coculture of derived epithelial cells and human primary vocal fold fibroblasts. Fibroblasts were seeded in a collagen I gel and left to contract for 2 days (**a**). Derived epithelial cells were seeded on the gel and submerged in media for 2 days (**b**). Media were removed from the construct surface and cells were cultured at the air–liquid interface for 3 weeks (**c**)
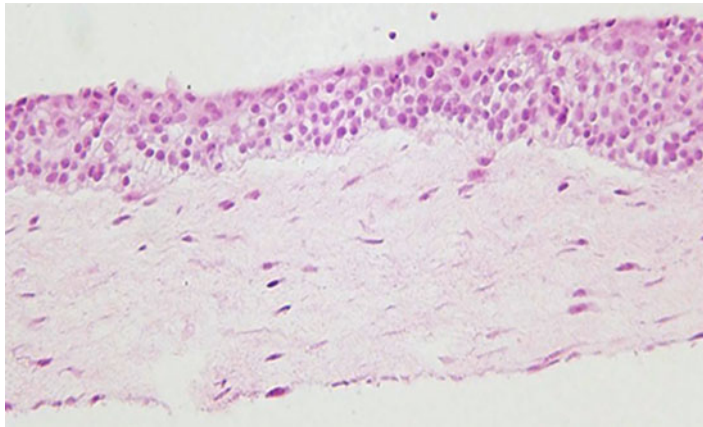
Fig. 2 Hematoxylin and eosin (H and E) staining of a representative engineered mucosa showed a multilayered, noncornified, squamous epithelium consistent with the vocal fold epithelium (200-µm scale bar)

3. Creating of Air–Liquid (ALI) interface:

   (a) Once the epithelial layer reaches confluence, which is approximately after 2 days, create an air–liquid interface.

   (b) Remove FAD media from the gel surface (Fig. 1) and change media around the inserts, add 1 ml FAD per well.

   (c) Cultivate cell at the ALI for 19–21 days with media changed round the insert every other day, add 1 ml FAD per well.

   (d) The final construct should demonstrate differentiated stratified squamous epithelial cell layers consistent with the native vocal fold epithelium as well as a complete and continuous basement membrane on a gel scaffold embedded with human fibroblasts (Fig. 2).

   (e) Vocal fold mucosa cultures are prepared for molecular, histological, and immunohistochemical analyses using standard procedures (Fig. 3). They can be also used for
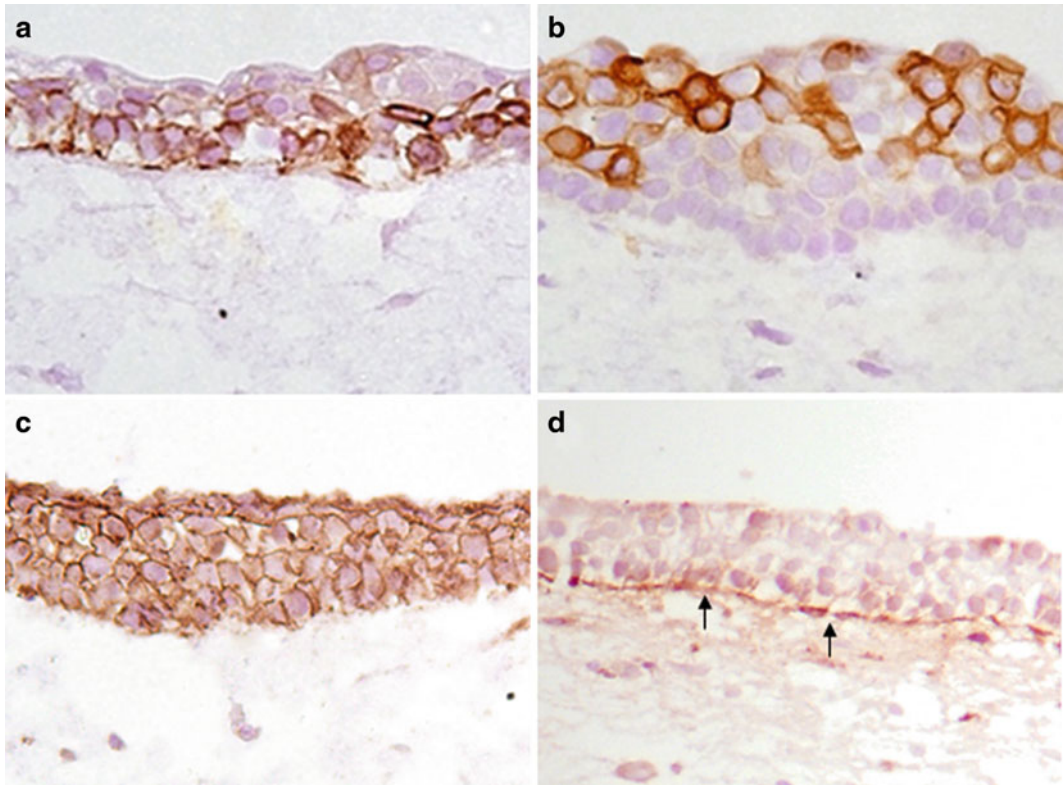
Fig. 3 Confirmation of the derived stratified squamous epithelium. Immunohistochemistry revealed positive staining for K14 in the deep layers of the epithelium (**a**), K13 in the suprabasal layers (**b**), and e-cadherin in all layers of the epithelium (**c**) consistent with the vocal fold epithelium. *Arrows* indicate positive staining for laminin-5 in the interface between the epithelium and the gel demonstrating the presence of a basement membrane (**d**)

functional experiments studying cell signaling pathways or modeling vocal fold damage and disease; and/or testing safety and effectiveness of novel cytoprotective and regenerative treatments in vitro.

4   Notes

1. Preparing Matrigel-coated plates must be done under sterile conditions and be performed as quickly as possible, as the Matrigel will gel rapidly at room temperature. Each Matrigel aliquot is intended for one use. Excess Matrigel may be plated and used within 7–10 days.

2. The thawing procedure must be performed as quickly as possible to ensure optimal cell recovery. It is recommended thawing

cells into a four-well plate to increase the chance of successful thaw and to help prevent contamination of the entire plate. Once successful thawing technique have been established, one vial of cells can be thawed directly into one well of a six-well plate.

3. During passaging the number of the cells expands. We recommend freezing down the cells which will not be used for further differentiation experiment. In freezing of hES cells follow the WiCell protocols (3).

Acknowledgement

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Human Embryonic and Hepatic Stem Cell Differentiation Visualized in Two and Three Dimensions Based on Serial Sections

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Abstract

Pluripotent human embryonic stem cells (hESCs) are characterized by two defining properties, self-renewal and differentiation. Self-renewing hESCs express transcription factors OCT4, SOX2, and NANOG, and surface markers SSEA-4 and TRA-1-60 and TRA-1-81 and their ability to differentiate into derivatives of the three germ layers show the differentiating potential. Studies suggest a certain microheterogeneity of the hESC colonies, in which not all cells in one colony of apparently undifferentiated cells express all the expected markers. We describe a technique to paraffin embed an entire hESC colony, and prepare 3–5 μm thick serial sections. Immunohistochemistry applied to individual sections produces a 2-dimensional survey of the developing hESC colony. Based on serial paraffin sections of the 2D-expression pattern, a new and useful 3D-visualization can be modeled. The actual 3D rendering of an entire colony is accomplished using 3D image processing software such as Mimics® or Amira®. An extended version of this technique even allows for a high-magnification 3D-reconstruction of, e.g., hepatic stem cells in developing liver. These techniques combined allow for both a 2- and a 3-dimensional visualization of hESC colonies and stem cells in organs, which leads to new insights into and information about the interaction of stem cells with their surroundings.

Keywords: 3D-reconstruction, Amira, hESC, Mimics, Pluripotency markers

1 Introduction

Pluripotent human embryonic stem cells (hESCs) were first successfully derived by Thomson and colleagues in 1998 (1) using the inner cell mass of pre-implantation blastocysts, and later in 2007 induced pluripotent stem cells, iPS cells, were established from human somatic cells (2, 3). The use of stem cells to mimic normal human development and to create specific disease models from certain organs, e.g., the liver, will lift our understanding of basic human development and disease mechanisms to another level. Expectations for their beneficial use for therapeutic purposes and regenerative medicine have fuelled a huge interest in understanding how controlled differentiation occurs and which cues determine specialization and cell function.

Pluripotent human embryonic stem cells are morphologically characterized by a high ratio of nucleus to cytoplasm and prominent nucleoli, and are characterized by two defining properties, self-renewal and differentiation (1, 4). Self-renewing capabilities are demonstrated through numerous passages of the undifferentiated cells while maintaining expression of pluripotent markers such as OCT4, SOX2, and NANOG, and surface markers such as stage-specific embryonic antigen 4 (SSEA-4), and tumor-recognition antigens TRA-1-60 and TRA-1-81 (1, 4–7). Validation of pluripotency is performed in vivo by formation of teratomas, containing tissue types representative of all three germ layers, after injection of undifferentiated cells into immunodeficient mice (4, 7, 8). Alternatively, the cells may be allowed to form embryoid bodies in vitro, which are spontaneously differentiated cells that comprise the three germ layers (5, 9). Traditionally, characterization is done by the "one marker—one colony"—approach in which an entire colony is used for the detection of a single marker. However, studies suggest a certain micro-heterogeneity of the expression of hESC markers (10, 11), which demonstrates that not all cells in a hESC colony of apparently undifferentiated cells express all the expected markers. In fact neighboring cells may express markers characteristic of totally different cell types (10), which at the macroscopic level may reflect the different shapes and patterns of hESC colonies. In order to characterize the differentiation processes that occur in hESC colonies and also in organs leading to more specialized cell types, an understanding of the starting point and the growth and development of the hESC colonies is needed. To this end, a 2- and 3-dimensional approach to detect and visualize expression of a desired number of markers of both undifferentiated and differentiated cells in individual colonies of hESCs or in stem cell areas of more mature organs is highly beneficial.

This chapter describes approaches to detect expression of a number of different markers of both undifferentiated and differentiated cells in individual colonies of hESCs and in developing human liver and digitally reconstructing the expression patterns in three dimensions (3D). Three-dimensional reconstructions can be divided into two categories: volumetric rendering and surface rendering through segmentation, respectively (12, 13). A volume can be divided into three-dimensional pixels termed "voxels." Volumetric rendering is the visualization of volumes through calculation of brightness and color from each voxel based on voxel color values and transparency (14). Segmentation based renderings, on the other hand, involves assigning each pixel in an image stack to a specific "label." Thereby, pixels assigned to the same label, such as bone or a cell type can be represented in three dimensions.

In this chapter two different techniques for 3D visualization are described. The first method includes a technique to paraffin embed and serial section an entire hESC colony (e.g., ~150 μm thick).

Immunohistochemical staining procedures applied to individual sections produce a 2-dimensional survey of the developing hESC colony. This 2D-expression pattern can be used to create a 3D-model of the culture. Photos of individually stained sections, processed using image editing software, for instance Adobe® Photoshop®, can be used to create a 3D visualization, in this approach with the 3D visualization software Mimics®. This technique generates new information about interaction of individual cells within a given stem cell colony.

Amira® is a powerful and modular 3D software platform that can manipulate and visualize biomedical data from a vast number of sources, including 2D, 3D, and volume data. However, Amira® may also be highly complicated to use and has a steep learning curve. The second approach presents a method in which the stem cell niche in approximately 250 μm of developing liver is reconstructed from serial sections (12). Many software solutions for 3D reconstruction only allow manipulation of grayscale images, thereby loosing information of color expression in photomicrographs. By combining Adobe® Photoshop® with Amira® the second approach to 3D visualization employ color enhancement and recognition in serial photomicrograph. Through these means the hepatic stem cell niche can be reconstructed through both volumetric and segmentation based rendering.

2   Materials

Generation of specific 3-dimensional reconstructions requires besides cells or tissue, specific technical equipment, and software.

2.1   Cell Culture and Derivation

Culture of hESCs requires access to sterile culture facilities including flow benches, microscopes, and incubators, and can normally be performed in a standard equipped culture laboratory.

2.2   Paraffin Embedding and Immuno-histochemistry

Only standard technical equipment, including microtome and microwave oven, of a histochemistry laboratory is needed. Graded alcohols, xylene, Histowax with melting point 56–58', standard buffers, and an antigen retrieval system are also required. Details about antibodies used in this chapter are listed in Table 1.

2.3   Special Requirements for 3D Modeling

1. Paraffin sections with similar slice thickness, e.g., 3 μm, preferably immunohistochemically stained with antibody of interest—unstained sections may however also be used for the 3D model.

2. Light microscopic photographs of sections at desired magnification (i.e., low for entire colony, higher for area of interest (AOI)).

Table 1
Details on commercially available antibodies and detection used in this chapter

Antigen	Manufacturer	Code nr.	Antibody-species	Antigen retrieval/dilution	
OCT 3/4	Santa Cruz	Sc-8629	Goat		1:100
OCT4	Abcam	Ab 19857	Rabbit	TEG pH 9	1:250
NANOG	R&D	AF 1997	Goat	Citrate pH 6	1:50
TRA-1-60	Chemicon	MAB 4360	Mouse		1:100
SSEA-4	Chemicon	MAB 4304	Mouse		1:75
SSEA-1/CD15	BD-PharMingen	555400	Mouse		1:150
HNF-3β	Santa Cruz	Sc-6554	Goat	TEG pH 9	1:100
CD 34	DakoCytomation	M 7165	Mouse		1:25
p63	Oncogene	OP 132	Mouse	TEG pH 9	1:100
SOX2	R&D	MAB 2018	Mouse	TEG pH 9	1:70
nestin	Chemicon	MAB 5326	Mouse		1:300
PAX6	Chemicon	Ab 5409	Rabbit		1:8000
CD68	DakoCytomation	M 0814	Mouse		1:400

End user must optimize immunohistochemical procedures

3. Image editing software such as Adobe® Photoshop®, which is used in the present study.
4. Advanced 3D image processing software such as Mimics® or Amira® used in the present study.
5. Preferably a pen tablet and pen as input-device instead of an ordinary mouse (e.g., a Wacom Board, Wacom Europe, Krefeld, Germany).

3 Methods

The procedure combines a special paraffin embedding technique, required for the 2-dimensional survey and the 3-dimensional reconstructions of hESC colonies, with immunohistochemistry, and enables the visualization of the regional distribution and co-localization of both embryonic stem cell and germ layer markers. The 3-dimensional reconstructions with special 3D-rendering software of either an entire colony or an AOI also require plotting of the used antibodies on the created figure in order to visualize the 3-dimensional distribution of the different cell types.

3.1 Derivation and Culture of hESCs

Surplus human embryos from couples undergoing IVF treatment donated for hESC derivation were used for establishment of hESC lines.

1. Embryos were grown to the blastocyst stage and the inner cell masses were manually isolated using 27G needles attached on 1 ml syringes under a stereomicroscope.

2. Each individual inner cell mass was plated onto a tissue culture dish pre-coated with mitotically inactivated human foreskin fibroblasts (hFF) or mouse embryonic fibroblasts and allowed to attach.

3. Colonies of outgrowing hESCs were manually isolated with hypodermic needles and passaged on fresh hFF once every 7–10 days. Areas with just a single confluent layer of cells that displayed the typical appearance of undifferentiated hESC as determined under the inverted microscope were chosen for passage.

4. One milliliter syringes attached with 27G needles were used to cut out clumps of cells containing around 100–200 cells. Four to eight clumps of cells were normally passaged from one culture dish to a new containing fresh feeder cells. The passage number increases with one for every passage and the day of transfer is designated "the starting point" or day 1 for that particular dish.

3.2   Paraffin Embedding

Colonies of four well-characterized hESC lines (LRB01-04) were grown on mouse embryonic feeder cells for 4, 11, 21, 28, and 30 days in hESC culture medium (*see* Laursen et al., 2007 for detailed information (10)). In an attempt to preserve the 2D structure of flattened colonies (**A**) and the 3D structure of the more differentiated—and thus bulging—colonies (**B**), individual samples were fixed and embedded using two different strategies depending on their appearance (Fig. 1).

1. **A**: Flat colonies were fixed in Bouin's fixative in situ in the culture dish (Fig. 1a). After 1–2 h of fixation the fixative was replaced with 70 % ethanol, and 24–48 h later the 70 % ethanol was replaced with 90 % ethanol.

2. Following overnight dehydration in 90 % ethanol, the samples were exposed to 99 % ethanol for 12 h.

3. Then the colonies were gently dissected free from the bottom using a Cell Scraper (Nunc) starting from the periphery. Colonies were then lifted carefully from the bottom of the culture dish to a small metal embedding mould into which xylene was pipetted.

4. After 1 h's exposure to xylene, paraffin was gently added to the embedding mould.
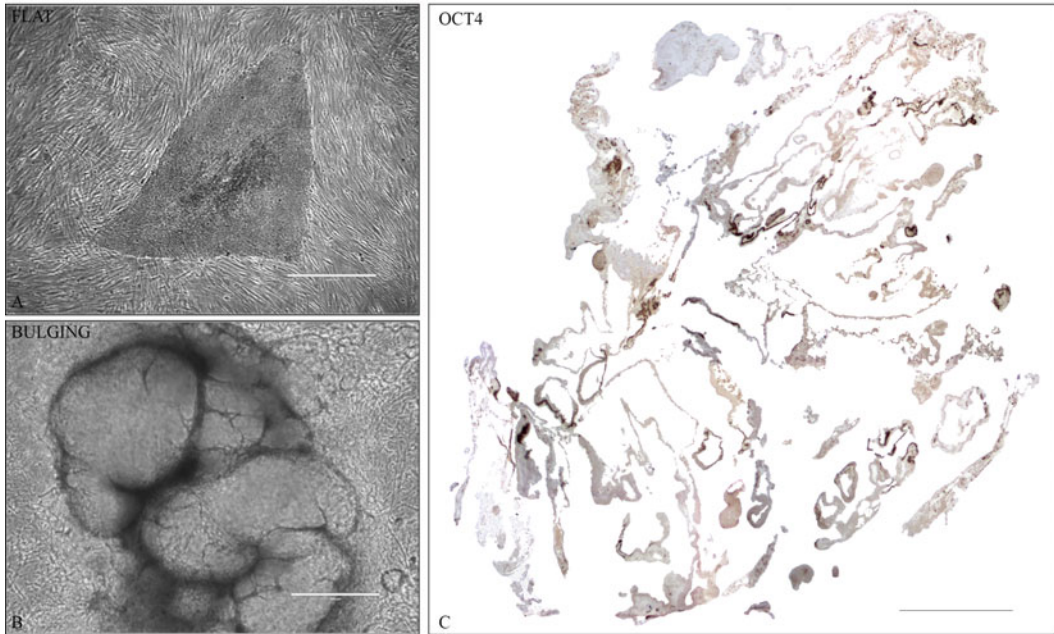
Fig. 1 Examples of (**a**) flat and (**b**) bulging hESC colonies prior to paraffin embedding. (**c**) Survey of a flat colony, OCT4 stained. Certain areas are specific for the OCT4 staining while other areas are found negative. Neighboring sections showed different groupings of cells based on the markers used, i.e., p63 areas were found, often in close connection to the OCT4 cells. Several times, there was a cell-type change from OCT4 positive to p63 positive on a one-cell basis. Section number 29 out of a total of 70, colony age is 32 days. Scale bar: 1 mm

5. **B**: Prior to fixation the more differentiated, bulging colonies intended for 3D analysis were carefully dissected free and lifted from the bottom of the culture dish using hypodermic needles (Fig. 1b).

6. Individual colonies were placed between two cover glasses and fixed in Bouin's fixative for 1–2 h followed by dehydration in graded alcohols.

7. Finally the colonies were cleared for 1 h in xylene and embedded in paraffin.

8. Embedded colonies were cut in 3–5 μm thick serial sections. Regional distribution of a panel of markers including antibodies against pluripotent hESCs (OCT4, NANOG, TRA-1-60, SSEA-4) as well as antibodies against the three germ layers (HNF-3β, CD 34, p63, SOX2, nestin, PAX6, CD68, and SSEA-1) (Table 1) was studied on neighboring sections of the differentiating colonies. An OCT4-staining from a flat colony is shown in Fig. 1c. In theory, any antibody of interest may be used. The end user must optimize standard immunohistochemical procedures.
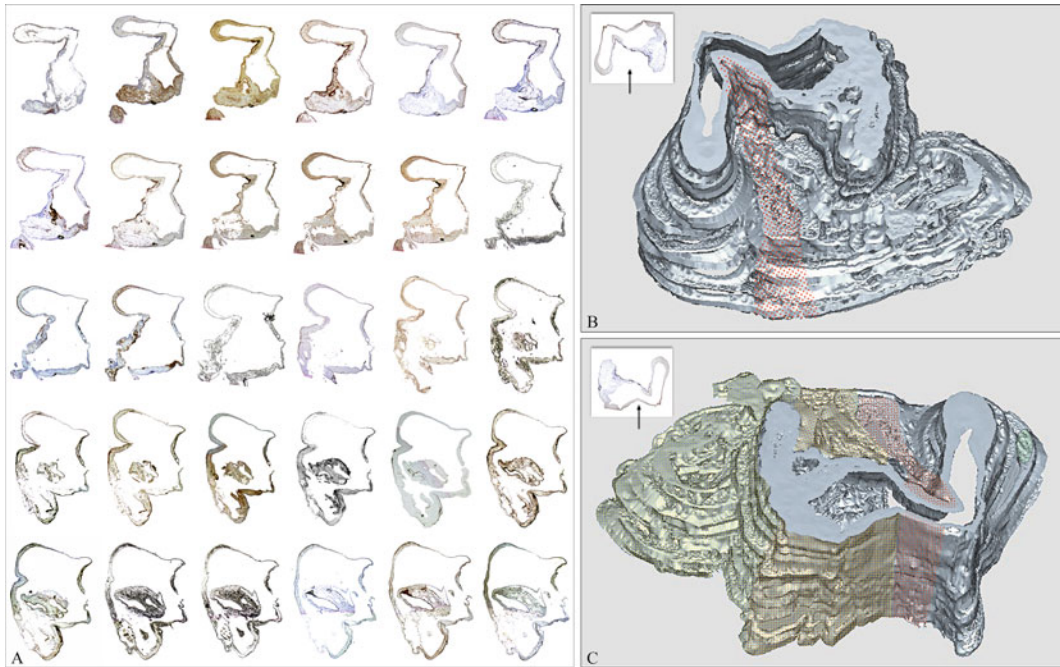
Fig. 2 Overview of sections used for generating a 3D model (**a**). Sections represent a bulging colony. Reconstruction of entire colony, seen from two angles (**b**, **c**). *Inserts* show direction of view and section level. *Red dots* correspond to human embryonic stem cells positive for markers for undifferentiated embryonic stem cells, such as OCT4, NANOG and TRA-1-60 (the "stem cell belt") while *yellow dots* correspond to a more differentiated area, positive for several known germ layer markers such as p63 (ectoderm), CD 34 (mesoderm) and HNF-3β (endoderm)

3.3 3-Dimensional Reconstruction of Entire Colony Using Mimics® 10.01 and Adobe® Photoshop®

3.3.1 Image Acquisition and Alignment

In order to create a 3-dimensional reconstruction of consecutive immunohistochemically stained paraffin-embedded sections, photomicrographs of the sections intended for the 3D model are saved in an appropriate digital file format such as Tagged Image File Format (TIFF). Save images in the same folder with consecutive numbering such as 001, 002, and 003 (a representation of sections from a bulging colony is shown in Fig. 2a). In order to align and edit the pictures of the paraffin sections on the digital canvas, image editing software, for instance Adobe® Photoshop® is required. Aligned images must be saved in a BMP-format and all images must have an equal size (e.g., 4.933 KB).

1. Alignment of serial sections closer to each other leads to a more accurate alignment compared to using sections far from each other (i.e., section #1, #4, #7, #10, etc. leads to a more accurate alignment than using section #1, #10, #20, #30, etc.) (*see* **Note 1**).

2. Initially it is recommended that all pictures intended for the 3D reconstruction be purified of undesired background colors using the eraser and lassotool. This should leave a picture only showing the cell colony.

3. Adjust canvas dimensions on all pictures to that of the largest picture.

4. Save the first slide in a BMP format, it is recommended to use filenames showing their numerical order (e.g., 001.bmp, 002. bmp, 003.bmp).

5. Set background as white and create a new image with these newly set dimensions. Create a new layer. This image works as a background picture.

6. Select the cleansed figure on slide #1 and copy this into the new layer on the background picture.

7. Select the next figure on the following slide and copy this selection into a new layer on the background picture.

8. Set transparency on this new layer to 60 %, which allows both layers to be visible at the same time. Position slide #2 exactly on top of slide #1, in order to recreate the cells' position before paraffin sectioning. Then reset transparency level to 100 %. Using a selected fix point on the images may help this repositioning (it is mandatory that the fix point remains consistent throughout the entire colony).

9. Delete the primary layer, flatten image and save this new image in numerical order, i.e., 002.bmp, if the previous image was saved as 001.bmp. Now restart from 7 with all images in the reconstruction library (*see* **Note 2**).

3.3.2 3-Dimensional Reconstruction of hESC Colony

1. Open Mimics, click on File and Import Images.

2. Find the saved images, and select all the images intended for the 3D figure. Mark the "Manuel Import" box in the bottom of the screen and click Next.

3. The "Manuel Conversion Options" screen now appears. Make sure that the desired images are shown on the left-hand screen (Multiple Files), and sorted in numerical order. This can be changed using the "Num" button. Set the image size to the correct number of pixels (information you can find either by right-clicking the image, and then Preferences or by getting image information using Photoshop). The next step is setting slice distance. The exact distance between sections can be very small, e.g., 3 μm, which will lead to a very flat 3D figure (depending on the total number of sections used). Hence, it is recommended to select a much higher value, e.g., 500 μm, leading to a 3D visualization that is more easily viewed. When desired slice distance is selected, click OK.

4. Make sure all images are selected on the next screen and press the Convert button.

5. A Mimics project is now created and opened, showing the Change Orientation window. Here the image orientation is

set (Anterior/Posterior, Top/Bottom, Left/Right). Click OK when adjusted.

6. The current project is now ready for modifications before the actual 3D calculation. First contrast and threshold needs to be set. These values are applied to all images and by scrolling through the image stack; the effect of the threshold value can be viewed on the individual images. Normally, the best pictures (with less holes or blank/white areas) intended for the reconstruction are obtained by selecting a low value for the lower threshold value and a high value for the upper threshold value.

7. Additional useful image editing can be done using the Segmentation menu. Here, tools such as Thresholding, Region Growing, Edit Masks, and Calculate 3D can be found. The Region Growing tool makes it possible to split the segmentation created by thresholding into several objects and to remove floating pixels. Click the Region Growing button, and select Source and Target mask. By clicking on one point in the source area (the cell culture), a segmentation calculation is commenced. All points in the current segmentation that are connected to the selected point will be used to form a new mask, given a new color (*see* **Note 3**).

8. To make this new mask active deselect the original mask (by clicking on the glasses next to it) and enable the new mask, by clicking on the glasses. Fine editing can now be done using this new layer. By reviewing the individual images, the effects of this new mask can be seen, and where needed further fine-tuning can be done before building the 3D representation.

9. All manual editing functions are performed on the active mask. Using the Edit Masks tool it is possible to draw, erase or restore the image with a certain threshold value. The tool can be set to either a circle or a rectangle and can have different sizes. On the newly created mask, adjustments can now be made (cells deselected during Region Growing can be re-drawn into the mask, and floating pixels can be deleted). This action should be performed on all images, leaving a final result of all colored cells.

10. On the mask tab, the selected mask is highlighted. Make sure that this is the mask containing the colored cells from the previous steps. Now, click on the calculate 3D-button either in the Segmentation Menu or in the Mask Tab. A Calculate 3D dialog will pop up, giving the choice of selecting quality. High quality will give long rendering time but a better 3D recreation. Press Calculate to start calculating the 3D. When done, the 3D recreation will automatically appear on the screen.

11. Depending on the number of markers used, these can now be added to the figure. This is done by adding a new mask (rename

it as the marker used), and on this new mask only highlight the cells positive for the marker (or markers, if it is a general undifferentiated stem cell area). The Edit Masks menu is useful for this purpose, and it should be carried out on all images involved.

12. Now calculate the 3D on this new layer. When done, both 3D figures are shown on the screen. By clicking the glasses next to either of these, they will be selected or deselected (shown/not shown) (Fig. 2b, c).

13. Using the Transparency tool in the 3D menu, it is possible to view cells deeper within the culture, which may well be positive for certain markers used. This can be toggled on or off as well as the 3D-figure can be rotated in all directions for further visualization.

14. Continue this procedure with as many layers as desired.

15. Press "*Save*." It is generally recommended to save the entire project at frequent intervals during the entire modeling process.

3.4   3-Dimensional Reconstruction of an Area of Interest in Serial Sections Using Amira® 5.2.0. and Adobe® Photoshop® CS6

Per default the Amira® platform display four "windows": to the left the "*Pool*" and "*Properties*" window and to the right the "*Graphics*" window and "*Console*" (*see* Fig. 3). The basic components of Amira® are data objects and modules. Where data objects represent data, such as images, modules are used for visualizing or performing computational operations on the associated data objects. Data objects and modules and represented by icons displayed in the "*Pool*." When an icon representing data objects or modules in the "*Pool*" is selected information regarding the icon and related tools for data manipulation are displayed in the "*Properties*" window. Data is visualized in the "*Graphics*" window and the "Console" will provide information of computational processes.

3.4.1   Image Acquisition and Alignment

1. Image acquisition: Photomicrographs of an area of interest (AOI) are saved in an appropriate digital file format such as the Tagged Image File Format (TIFF). Save images in the same folder with consecutive numbering, e.g., 00, 01, 02, and 03.

2. Import images into Amira: Start Amira® and press "*Open Data*" in the "*Pool*." Navigate to the folder containing the images; select all relevant images and press "load". In the appearing "*Image Read Parameters*" dialogue box keep the default parameters including "*ColorField*" in the "*Channel Conversion*" box and press "*ok*." "Right-clicking" on the resulting image stack data object in the "*Pool*" will allow addition of the modules "*OrthoSlice*" and "*BoundingBox*" to the image stack. These modules permit visualization of the consecutive images and outline the border of the image stack in the
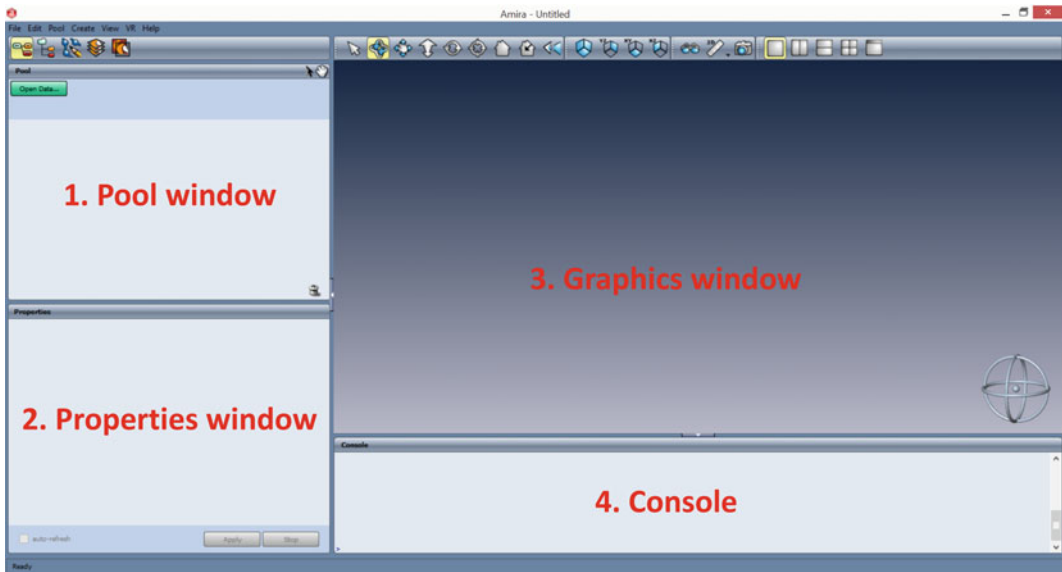
Fig. 3 Presentation of the Amira platform. The default Amira platform displays four windows; (1) The "Pool," (2) "Properties," (3) the "Graphics" window, and (4) the "Console." "Objects" and "Modules" are displayed in the "Pool." Information and related tools for selected "Objects" are displayed in the "Properties" window. Visualizations of "Objects" are displayed in the "Graphics" window. The "Console" provides information of related computational processes

"*Graphics*" window, respectively. A run through the image stack is possible by sliding the "slice number" tool in the "*Properties*" window for the "*OrthoSlice*" module.

3. Alignment of images: Right click on the data object representing the image stack in the "*Pool*" window → choose "*Compute*" → "*AlignSlices.*" Choose "*Edit*" in the "*Properties*" window for the "*AlignSlices*" module and the image stack will be presented in the "*Graphics*" window. Two semitransparent neighboring images will be presented in an overlaid fashion at a time, one image with colors inverted. Use the computer mouse to drag and turn the position of one image relative to the other in order to conduct "neighborhood alignment" (*see* **Note 4**).

4. Having aligned two images use the slice number slider at the top of the "*Graphics*" window to proceed and repeat the procedure with each consecutive pair of images.

5. When finished press "*Resample*" in the "*Properties*" window for the "*AlignSlices*" module. A new data object representing the aligned images will appear in the "*Pool.*"

6. Press "*File*" → "*Save Network As*" in order to save the created network of data objects and connecting modules. Throughout
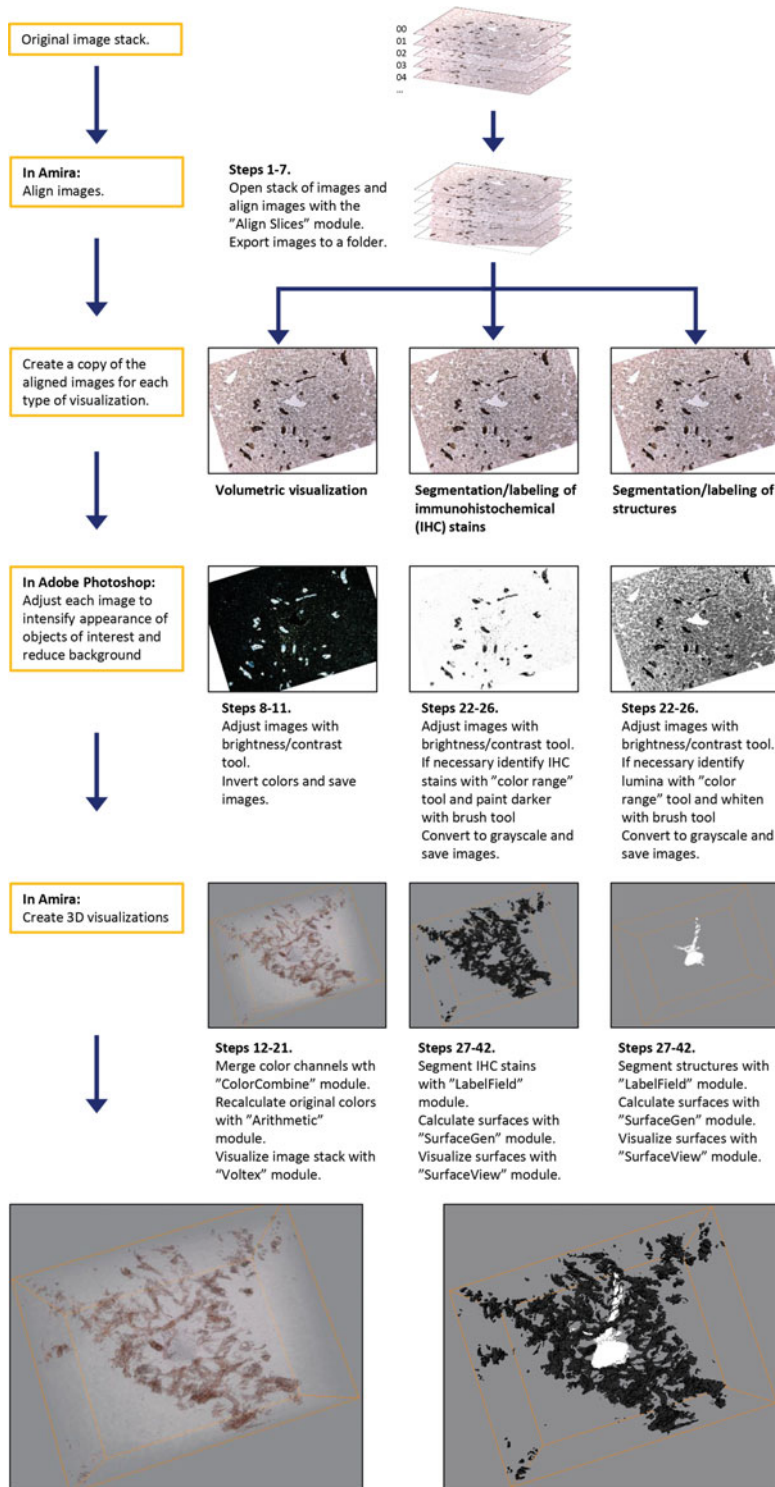
Fig. 4 Overview of processes for volumetric and segmentation/labeling based 3D-renderings of serial sections using Amira. Serial sections of a tissue are photomicrographed. Steps 1–7: Photomicrographs are aligned with respect to each other in Amira and exported. Copies of the aligned photomicrographs are created for

this protocol, the network should be saved frequently, in order to minimize loss of data due to computer error.

7. Choose the data object representing the aligned images and press "*File*" → "*Save Data As.*" Choose a folder where to save the aligned images and save them in an appropriate file format, e.g., in 2D TIFF. Close Amira®.

3.4.2 Volumetric 3D Rendering (See Fig. 4)

The aligned images are saved in the chosen folder. Create a copy of the entire folder for the volumetric 3D rendering and a folder for each category of structure that will be reconstructed in 3D. With this approach one copy of the aligned images can be used for segmenting structures and another copy can be used for segmenting immunohistochemical stains.

Preparation of images for volumetric rendering: With volumetric rendering, the images are displayed as they appear in the photomicrograph. However, in order to see through the image stack in 3D it is necessary to adjust each image so unwanted background is reduced and the visual appearance of objects of interest is intensified.

8. Open each aligned image contained in the folder for volumetric rendering in Adobe® Photoshop®.

9. Press "*Image*" → "*Adjustments*" in the top panel. Choose a suitable tool for image adjustment, for instance "*Brightness/ Contrast.*" Use the sliders in this tool to reduce background and increased darkness of immunohistochemical stains.

10. Press "*Image*" → "*Adjustments*" and choose "Invert" to invert the colors of each image.

Following their preparation in Adobe® Photoshop® the inverted images are loaded into Amira®. In Amira® the images are split into their color channels. The color channels are recalculated in order to reinvert the images. This step renders the background of photomicrographs transparent.

Fig. 4 (continued) volumetric visualization and segmentation/labeling of immunohistochemical stains or structures, respectively. Steps 8–11 and 22–26: Photomicrographs are adjusted in Adobe Photoshop to intensify the appearance of the objects of interest while reducing background. Finally, photomicrographs optimized for volumetric rendering are color inverted while photomicrographs optimized for segmentation/ labeling-based 3D reconstruction are converted into grayscale images. Steps 12–21: Photomicrographs optimized for volumetric rendering are loaded into Amira. In Amira, the color channels are merged with the "ColorCombine" module and the original colors are recalculated with the "Arithmetic" module. The "Voltex" module is used for visualizing the resulting image stack. Steps 27–42: Photomicrographs optimized for segmentation/labeling-based 3D reconstruction are loaded into Amira. The immunohistochemical stains or structures of interest are labeled using the "LabelField" module. The surfaces of each label are then calculated with the "SurfaceGen" module. Finally, the calculated surfaces are visualized with the "Surface-View" module

11. Save each image in, for instance, the "Joint Photographic Experts Group" (JPEG) format.

12. In Amira®: Press "*Open Data*" and load the aligned color inverted images simultaneously. Choose the "*All Channels*" option in the "*Image Read Parameters*" dialogue box and press "*ok.*" The resulting image stack is displayed as a "*data object*" in the "*Pool*" window with each color channel represented as separate data objects attached.

13. Right click on one of the color channels → press "*Compute*" → "*ColorCombine.*"

14. Left click in the white square in the resulting "*ColorCombine*" module and add another color channel as "*Source2*" simply by clicking on the color channel data object.

15. Repeat the above step and add a third color channel as "*Source3.*"

16. In the "Properties" window for the "ColorCombine" module choose "*RGB Planes*" in the "*Color Mode*" section and press "*Apply.*" A "*Combination*" data object will then appear in the "*Pool*" window representing the three combined color channels.

17. Right click the "*Combination*" data object → press "*Compute*" → "*Arithmetic.*" The resulting "*Arithmetic*" module allows calculations to be conducted on each color channel.

18. In the properties window for the "*Arithmetic*" module choose "*like input A*" in the "*Result channels*" section. The three sections beneath, "*Expr R,*" "*Expr G,*" and "*Expr B*" represent the color channels red, green and blue, respectively. "*Expr A*" represent "transparency."

19. In the "*Expr R*" section enter: Ar*-1 + 255. In the "*Expr G*" section enter: Ag*-1 + 255. In the "*Expr B*" section enter: Ab*-1 + 255. In the "*Expr A*" section enter: Aa. In the "*Result type*" section choose "*input A*" and press "*Apply.*" These steps reinvert the colors to the original.

20. In order to visualize the computed "*Result*" right click on the data object "*Result*" in the "*Pool*" → press "*Display*" → "*Voltex.*" The image stack volume will be displayed in the "*Graphics*" window. The volume can be rotated and zoomed in and out through use of the tools presented in the "*Graphics*" window. Alternatively, the same procedures can be carried out using the computer mouse buttons and scroll wheel.

21. In the "*Properties*" section for the "*Voltex*" module the transparency of the volume in the "*Graphics*" window is adjustable by sliding the "*Alpha Scale*" tool.

3.4.3 Segmentation-Based 3D Rendering
(See Fig. 4)

Preparation of images for segmentation-based 3D rendering: This form of rendering is based on identifying structures or objects of interest on photomicrographs and assigning them to different "labels." Identifying and outlining objects of interest is relatively simple on color photomicrographs. However, the "labeling" or "segmentation" process in Amira and many other 3D-software platforms is carried out on grayscale images. This results in images where objects of otherwise different colors may be difficult to distinguish when displayed in nuances of gray. Therefore, it may be necessary to enhance the visual appearance of objects of interest on color photomicrographs prior to their conversion to grayscale images, thereby increasing the signal-to-noise ratio. In the section beneath is described a procedure for segmenting colors from immunohistochemical staining.

1. Locate the previously created folder containing aligned images for segmentation and load the images into Adobe® Photoshop®.

2. Press "*Image*" → "*Adjustments*" in the top panel. Choose a suitable tool for image adjustment, for instance "*Brightness/ Contrast*." Use the sliders in this tool to reduce background and increase darkness of immunohistochemical stains.

3. Other tools may be applied such as the "Color Range" tool ("*Select*" → "*Color Range*") to identify specific colors followed by the "*Brush Tool*" to either darken these colors or to remove them.

4. Convert images to "grayscale": Press "*Image*" → "*Adjustments*" → "*Grayscale*" in the top panel.

5. Save each image in, for instance, the JPEG format.

Following their preparation in Adobe® Photoshop® the images are loaded into Amira. In Amira, the images are manually and/or semiautomatically segmented. The surfaces of each segmented or "labeled" structure of interest is finally calculated and displayed.

6. In Amira®: Press "*Open Data*" and load the aligned grayscale images simultaneously.

7. In the appearing "*Image Read Parameters*" dialogue box keep the default parameters, including "*Channel 1*" in the "*Channel Conversion*" box and press "*ok*." In order to add "depth" to the image stack, i.e., increase the "Z" axis, the numeric size of the "bounding box" may be exaggerated in the "*Image Red Parameters*" dialogue box.

8. The resulting image stack is displayed as a "data object" in the "Pool" window.

9. Segmentation: Right-click on the image stack data object → choose "*Labeling*" → "LabelField."

In "*LabelField*" mode the Amira® workspace will change to an environment designed for labeling structures of interest on the loaded images. The former "*Pool*" window will display each label created by the user and options for visualizing and modifying the given labels. The "*Properties*" window will now present a range of tools for defining/labeling the structures of interest. The "*Graphics*" window displays the consecutive grayscale images and the outlined labels. Tools in the "*Properties*" window can be used for directly defining and outlining structures on the images displayed in the "*Graphics*" window.

10. Create a label: In the "*Pool*" window go to the "*Materials*" option and press "*New.*" A new label named "*Material*" followed by a number will appear in the panel beneath. Double click on the "*Material*" text in order to rename it to, for instance, "*Cytokeratin 19*" and press "*enter*" on the keyboard.

11. Left click on the label that is to be edited, for instance, "*Cytokeratin 19.*"

12. Choose a tool in the "*Properties*" window for outlining the label in the "*Graphics*" window. The most basic tool is the "*Brush*" tool.

13. With the "*Brush*" tool activated go to the "*Graphics*" window. Outline the cytokeratin 19 staining on the image by holding down the left mouse button and moving the brush. When the labeling process of the image is finished choose "*current slice*" in the "*Pool*" and press "+" in order to add the label to the image.

14. This tedious process can be accelerated using the "masking," "*lasso*," "*magic wand*," "*thresholding*," or "*region growing*" tools. Having labeled two relatively distant images, the images in between can also be automatically labeled through interpolation.

15. Go to the next image in the series by pressing the "*space bar*" or "*arrow*" keys on the keyboard and repeat the labeling process (*see* **Note 5**).

16. When the labeling process is finished save the labels in a separate file. In the top panel go to "*File*" → "*Save Data As.*" Choose a destination, for instance the folder containing the grayscale images, name the file, and save the label in the AmiraMesh RLE (am) file format.

17. In order to visualize the label go back to the original work environment by clicking on the "*Object Pool*" icon in the upper left corner.

18. In the "*Pool*" window a data object containing the label is now added to the image stack data object.

19. Visualize the label by right clicking on the label data object → choose "*SurfaceGen.*"

20. Click on the "*SurfaceGen*" module, choose whether the resulting surface should be smooth or not and press "*Apply.*" A new data object containing the calculated surfaces will appear in the "*Pool.*"

21. Visualize the surfaces by right clicking on the surface data object → choose "*SurfaceView.*" The surfaces are now displayed in the "*Graphics*" window where they can be rotated and zoomed upon (*see* **Note 6**).

4   Notes

1. In case the entire colony should be re-created, and the light microscopic camera cannot contain the entire colony in one picture, it is possible to divide the colony into multiple pictures and then re-combine them using the Photomerge function in Photoshop®. When doing this, it is important to retain overlapping regions in the pictures in order to recombine these correctly. However, the best result is obtained by scanning the entire slide using a Slide Scanner.

2. It is advisable to save one image containing layers. When alignment is done with the newly added layer, flatten image and save this as the next BMP image. Then undo the flatten-image action, delete the older of the two layers and then add the next layer. This should facilitate the alignment process.

3. Region growing alone may not be sufficient if all cells are not all connected to each other. Instead, an area around the cells can be selected and a region growing can be performed using this selection.

4. Neighborhood alignment is based on matching consecutive sections as well as possible. However, this alignment may not be accurate. It may be advantageous to embed fixed reference points (fiducial markers) into the paraffin-block in order to increase accuracy of the alignment.

5. If the objects to be labeled are well defined with clearly different nuances of gray segmentation can be greatly accelerated simply be adding the "IsoSurface" module to the image stack. This tool allows segmentation through defining a grayscale intensity threshold for each object of interest.

6. Displaying image stacks put great demands on computational power and memory. Adding the "resample" module to the image stack in order to reduce resolution can reduce these demands. A reduction in image resolution can also be completed in Adobe® Photoshop®.

Acknowledgments

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Derivation of Chondrogenic Cells from Human Embryonic Stem Cells for Cartilage Tissue Engineering

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Abstract

Human embryonic stem cells (hESCs) have the ability to self-renew and differentiate into any cell lineage of the three germ layers, therefore holding great promise for regenerative applications in dentistry and medicine. We previously described a micromass culture system as a model system to induce and study the chondrogenic commitment of hESCs. Using this system, chondrogenic cells can be further isolated and expanded under specific growth factor conditions. When encapsulated in hyaluronic acid (HA)-based hydrogels and cultured under appropriate growth factor and medium conditions, these chondrogenic cells synthesized and deposited extracellular matrix (ECM) characteristic of neocartilage. Here, we describe the micromass culture of hESCs, the isolation and expansion of hESC-derived chondrogenic cells, and the three-dimensional (3-D) culture of the chondrogenic cells in hydrogels for cartilage tissue engineering. We will also describe the various tools and techniques used for characterizing the tissue-engineered cartilage.

Keywords: Chondrogenic differentiation, Embryonic stem cells, Chondrocytes, Cartilage, Hydrogels, Tissue engineering

1 Introduction

Human embryonic stem cells (hESCs), derived from the inner cell mass of the blastocyst stage embryos, represent a promising cell source for transplantation because of their unlimited self-renewal and ability to differentiate into various cell lineages of the three germ layers (ectoderm, endoderm, and mesoderm) (1). To date, several diverse cell types have been derived from hESCs, including cardiomyocytes (2), endothelial cells (3–5), chondrocytes (6–8), osteoblasts (9, 10), neurons (11), keratinocytes (12), and hepatocytes (13).

Our group has previously established an effective high-density micromass system that induces chondrogenic commitment of embryoid body (EB) derived cells (14, 15). This chapter presents the improved protocol for chondrogenic differentiation of hESCs and isolation of putative chondrogenic cells for functional cartilage tissue engineering using hydrogels (16). A schematic illustration of the workflow is depicted in Fig. 1. Various analytical methods including biochemical and histological assays will also be presented.
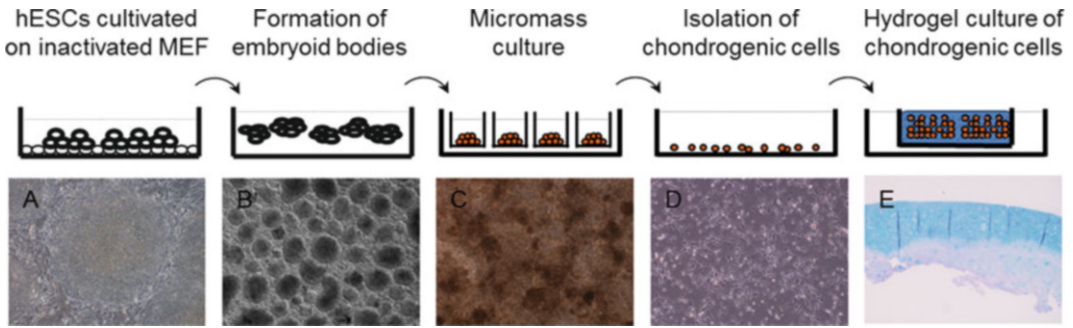
Fig. 1 Cultivation and differentiation of hESCs in vitro. (**a**) To keep hESCs undifferentiated and pluripotent, the cells were co-cultivated with growth-inactivated murine embryonic fibroblasts (MEF) in the presence of FGF-2. (**b**) After collagenase IV treatment, hESCs were cultured in suspension to form embryoid bodies (EBs) for a period of 5 days. (**c**) 5'd' EBs were dissociated into single cells and cultured at a high density of 3×10^5 cells per 15 μL spot in a 24-well plate pre-coated with 0.1 % gelatin. (**d**) hESC-derived chondrogenic cells were isolated and plated on collagen II-coated plates for further expansion. (**e**) hESC-derived chondrogenic cells were encapsulated in HA-based hydrogels at high density (2×10^6 cells/100 μL construct) and cultured in chondrogenic medium (medium 4 supplemented with 100 ng/mL BMP-7 and 10 ng/mL TGF-β1) for up to 28 days to form a cartilage tissue construct that is enriched in s-GAG as detected by Alcian blue staining

2   Materials

2.1   Cell Culture

1. Dulbecco's Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS) (Gibco/BRL).

2. Collagenase Type IV, Lyophilized (Cat No. 17104-019; Gibco/BRL, Gaithersburg, MD): Prepare the collagenase IV splitting medium (1 mg/mL) with DMEM/F12 medium. Filter-sterilize before use.

3. Collagenase P. (Cat. No. 11 213 857 001, Roche): Prepare the collagenase P splitting medium (1.5 mg/mL) (0.15 %) with DMEM/10 % FBS medium. Filter-sterilize before use.

4. 0.25 % trypsin/1 mM ethylenediaminetetraacetic acid (EDTA) solution (Cat No. 25200-072; Gibco/BRL).

5. 0.05 % trypsin/0.53 mM EDTA solution (Cat No. 25300-054; Gibco/BRL).

6. Cell dissociation buffer (Cat No. 13151-014; Gibco/BRL).

7. Dimethyl sulfoxide, Hybrid-Max™ (DMSO) (Cat No. D2650; Sigma).

8. 15 and 50 mL falcon tubes (BD Biosciences Inc., Franklin Lakes, NJ).

9. 6- and 24-well Ultra-Low Attachment Microplates (Cat No. 3471 and 3473; Costar® Corning, Nagog Park Acton, MA).

10. 6-, 12-, and 24-well plates (Nunc, Wiesbaden, Germany).

11. Milli-Q-purified water (Millipore, Billerica, MA, USA).

12. Gelatin, Type A, From Porcine Skin, Approximately 300 Bloom (Cat No. G1890; Sigma). 0.1 % (w/v) gelatin-coated tissue culture plates. Weigh out 0.5 g gelatin in an autoclavable bottle and add 500 mL of distilled water (dH$_2$O). Autoclave with cap loosely tightened, allow cooling to room temperature (RT), and pipet into the culture plates. Coat the plates overnight by incubating at 37 °C until use.

13. Collagen, From Chicken Sternal Cartilage, Miller type II (Cat No. C9301; Sigma). Dissolve the collagen in acetic acid solution (pH 3) to obtain 5 mg/mL stock concentration. Dilute the stock solution 50-fold with sterile dH$_2$O to obtain a working concentration of 0.01 %. Coat the plates overnight by incubating at 37 °C until use.

14. 40-μm nylon cell strainer (Falcon Cat No. 352340; BD Biosciences).

15. 10 mL syringes (Becton Dickinson Inc.)

16. 22-G needles (Sterican B BRAUN).

17. Cyrovials (Nunc).

18. Cell freezing container (Nalgene).

19. Analytical Balance (Mettler-Toledo Pte Ltd).

20. Cell Culture Incubator (37 °C, 5 % CO$_2$).

21. Centrifuge (Eppendorf AG, Hamburg, Germany).

2.1.1 Medium

1. Dulbecco's Modified Eagle's Medium (DMEM)/F-12 medium (Cat No. 11330-032; Gibco/BRL).

2. DMEM high glucose (Cat No. D1152, Sigma, St Louis, MO, USA).

3. Knockout™ Serum Replacement (KSR) (Cat No. 10828-028; Gibco/BRL).

4. Fetal Bovine Serum (FBS) (Cat No. CH30160.03; Hyclone, Logan, UT).

5. ITS^{+1}; 100× (6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 ng/mL selenium, 1.25 mg/mL bovine serum albumin, 5.35 μg/mL linoleic acid) (Cat No. 354352; BD Bioscience Inc, Franklin Lakes, NJ).

6. 2-Mercaptoethanol, 14.3 M (Cat No. M7522; Sigma).

7. GlutaMAX™-I Supplement, 200 mM; 100× (Cat No. 35050-061; Gibco/BRL).

8. MEM Non-essential Amino Acids (NEAA) Solution, 10 mM; 100× (Cat No. 11140-050; Gibco/BRL).

9. Sodium pyruvate solution, 100 mM; 100× (Cat No. 11360-070; Gibco/BRL).

10. Penicillin/Streptomycin (P/S), 10,000 U/10,000 μg; 100× (Cat No. 15140-122; Gibco/BRL).

11. Bovine serum albumin (BSA) (Cat No. A9418; Sigma).

12. L-proline (Cat No. P5607; Sigma).

13. Ascorbic acid 2-phosphate (AA2P, Cat No. A8960; Sigma) 100× stock: dissolve 50 mg in 10 mL PBS. Aliquot in working volumes and store at −20 °C.

14. Dexamethasone (Cat No. D2915; Sigma) 1,000× stock: Dissolve 0.0115 g in 20 mL Milli-Q-purified water. Aliquot and store at −20 °C (*see* **Note 1**).

15. Medium 1: DMEM high glucose supplemented with 10 % FBS, 10 % KSR, 1 % sodium pyruvate, 1 % NEAA, and 100 U/100 μg P/S.

16. Medium 2: DMEM high glucose supplemented with 1 % ITS^{+1} (6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 ng/mL selenium, 1.25 mg/mL bovine serum albumin, 5.35 μg/mL linoleic acid), 1 % KSR (*see* **Note 2**), 2 mM GlutaMAX™, 40 μg/mL L-proline, 50 μg/mL AA2P, 1 % sodium pyruvate, 1 % NEAA, 10^{-7} M dexamethasone, and 100 U/100 μg P/S (*see* **Note 3**).

17. Medium 3: DMEM high glucose supplemented with 10 % FBS, 1 % sodium pyruvate, 1 % NEAA, and 100 U/100 μg P/S.

18. Medium 4: DMEM high glucose supplemented with 1 % ITS^{+1} (6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 ng/mL selenium, 1.25 mg/mL bovine serum albumin, 5.35 μg/mL linoleic acid), 2 mM GlutaMAX™, 40 μg/mL L-proline, 50 μg/mL AA2P, 1 % sodium pyruvate, 1 % NEAA, 10^{-7} M dexamethasone, and 100 U/100 μg P/S.

2.1.2 Growth Factors   The effects of growth factors on expansion and differentiation of hESC-derived chondrogenic cells were studied, with the source, preparation, and concentration used given in parentheses (*see* **Note 4**).

1. Recombinant human transforming growth factor-beta-1 (TGF-β1) (Cat No. 240-B; R&D Systems, Minneapolis, MN). Prepare 1,000× stock solution (10 μg/mL) by reconstituting in filter-sterilized 4 mM HCl containing 0.1 % (w/v) BSA, followed by freezing in aliquots of working volume. The final concentration used is 10 ng/mL.

2. Recombinant human bone morphogenetic protein 2 (BMP-2) (Cat No. 355-BM; R&D Systems) Prepare 1,000× stock solution (100 μg/mL) by reconstituting in filter-sterilized 4 mM HCl containing 0.1 % (w/v) BSA, followed by freezing in aliquots of working volume. The final concentration used is 100 ng/mL.

3. Recombinant human bone morphogenetic protein-7 (BMP-7) (Cat No. 354-BP; R&D Systems). Prepare 1,000× stock solution (100 µg/mL) by reconstituting in filter-sterilized 4 mM HCl containing 0.1 % (w/v) BSA, followed by freezing in aliquots of working volume. The final concentration used is 100 ng/mL.

4. Recombinant human insulin growth factor-1 (IGF-1) (Cat No. 291-G1; R&D Systems). Prepare 1,000× stock solution (100 µg/mL) by reconstituting in filter-sterilized PBS containing 0.1 % (w/v) BSA, followed by freezing in aliquots of working volume. The final concentration used is 100 ng/mL.

5. Recombinant human growth differentiation factor-5 (GDF-5) (Cat No. 120-01; Peprotech, Rocky Hill, NJ). Prepare 1,000× stock solution (100 µg/mL) by reconstituting in filter-sterilized PBS containing 0.1 % (w/v) BSA, followed by freezing in aliquots of working volume. The final concentration used is 100 ng/mL.

6. Recombinant human fibroblast growth factor-2 (FGF-2) (Cat No. PHG0021; Gibco/BRL). Prepare 1,000× stock solution (5 µg/mL) by reconstituting in filter-sterilized PBS containing 0.1 % (w/v) BSA, followed by freezing in aliquots of working volume. The final concentration used is 5 ng/mL.

7. Recombinant human platelet-derived growth factor-bb (PDGF-bb) (Cat No. 220-BB; R&D Systems). Prepare 1,000× stock solution (10 µg/mL) by reconstituting in filter-sterilized 4 mM HCl containing 0.1 % (w/v) BSA, followed by freezing in aliquots of working volume. The final concentration used is 10 ng/mL.

2.1.3 3-D Hydrogel Culture

Hystem®-C Hydrogel Kit (Cat. No. GS313P; ESI BIO—BioTime, Inc. Alameda, CA) containing Glycosil® (thiol-modified hyaluronic acid), Gelin-S® (thiol-modified gelatin), Extralink® (polyethylene glycol diacetate, PEGDA), and DG water (degassed deionized water).

1. 24-well transwell inserts and companion plates (Corning).

2.2 Biochemical Assays

2.2.1 Sulfated Glycosaminoglycan and DNA Assays

1. Papain digestion buffer: Dissolve 125 µg/mL papain (Cat. No. P4762, Sigma) in PBS with 5 mM cysteine hydrochloride (Cat. No. C6852, Sigma) and 5 mM Na_2EDTA. Filter-sterilize prior to use. Always prepare freshly every time.

2. Blyscan sulfated glycosaminoglycan (s-GAG) assay kit (Biocolor, Newtownabbey, Ireland) containing Blyscan Dye (1, 9-dimethyl-methylene blue), Dissociation Reagent, Reference Standard (bovine tracheal chondroitin 4-sulfate, 100 µg/mL).

3. Quant-iT Picogreen dsDNA Assay Kit (Molecular Probes, Cat. No. P7589) containing PicoGreen dye reagent (1 mL solution in DMSO), 20× TE (25 mL of 200 mM Tris–HCl, 20 mM EDTA, pH 7.5), and Lambda DNA standard (1 mL of 100 μg/mL in TE). Prepare 1× TE buffer in DNase-free water.

4. Multichannel pipette (Eppendorf AG).

5. Plate reader (Safire, Tecan, Austria).

6. 96-well black microplates (Corning).

2.3   Histological Processing and Staining

1. Fixative: 10 % buffered formalin (Sigma).

2. Ethanol (99.5 %). Prepared graded dilutions of 70, 80, and 95 % (v/v) of ethanol in dH$_2$O.

3. Alcian Blue working solution (0.5 % w/v): Dissolve 0.5 g Alcian Blue 8GX (Sigma cat no. A3157) in 100 mL 0.1 M hydrochloric acid solution (pH 1.0).

4. Mayer's hematoxylin (Sigma).

5. Nuclear fast red solution (Sigma).

6. Scott's tap water: Dissolve 3.5 g sodium bicarbonate and 20 g magnesium sulfate in 1 L dH$_2$O.

7. Differentiation solution: 10 mL concentrated hydrochloric acid (HCl) diluted in 1 L of 70 % (v/v) ethanol.

8. Paraffin (Leica Biosystems, Nussloch GmbH Heidelberger, Germany).

9. Xylene (VWR International, Poole, Dorset, UK).

10. DePex mounting medium (Cat No. 361254D; VWR International).

11. Superfrost® Plus microscope slides (Menzel-Glaser, Braunschweig, Germany).

12. ImmEdge hydrophobic barrier pen (Cat No. H-4000; Vector Laboratories, Burlingame, CA).

13. Filter paper (Whatman).

14. pH meter (Mettler-Toledo Pte Ltd).

15. Tissue processor (Leica Biosystems).

16. Paraffin embedding machine (Leica Biosystems).

17. Microtome (Leica Biosystems).

2.4   Immunohistochemical Staining

1. Fixative: 10 % buffered formalin.

2. Ethanol (99.5 %). Prepared graded dilutions of 70, 80, and 95 % (v/v) of ethanol in dH$_2$O.

3. UltraVision HRP Detection System (Cat No. TM-125-HL; Lab Vision Inc., Fremont, CA) containing Ultra V Block,

Table 1
Protocol for preparing a DNA standard curve

Standard No.	Volume (µL) of DNA standard (2 µg/mL)	TE buffer (µL)	Volume (µL) of working Picogreen® reagent	Final concentration (ng/mL) of DNA standards in Picogreen® assay
Blank	0	100	100	0
1	1	99	100	10 50 100
2	5	95	100	50
3	10	90	100	100
4	25	75	100	250
5	50	50	100	500
6	75	25	100	750
7	100	0	100	1,000

Biotinylated Goat Anti-mouse IgG, Streptavidin Peroxidase, Diaminobenzidine (DAB) substrate, and DAB chromogen (*see* **Note 5**).

4. Pepsin (Cat No. AP-9007-005; Lab Vision).

5. Hydrogen peroxide block (Cat No. TA-125-HP; Lab Vision).

6. Dulbecco's Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS).

7. Monoclonal anti-type II collagen antibody, clone II-II6B3 (Chemicon Inc., Temecuela, CA).

8. Monoclonal anti-type I collagen antibody, clone COL-1 (Sigma).

9. Monoclonal anti-type X collagen antibody, clone X-53 (Quartett Immunodiagnostika GmBH, Berlin, Germany).

10. Control mouse IgG_1 isotype (Invitrogen). The sources of antibodies and dilutions used are summarized in Table 1.

3 Methods

The methods pertaining to the formation of embryoid bodies (EBs) from hESCs, chondrogenic differentiation of EBs, isolation and expansion of chondrogenic cells, encapsulation of chondrogenic cells in hydrogels (*see* **Note 6**), and biochemical and histological analysis of cartilage-specific matrix proteins are described below:

3.1 Embryoid Body Formation

1. By day 6–7 of hESC culture (*see* **Note 7**), hESCs are allowed to culture in suspension to form embryoid bodies (*see* Fig. 1a, b).

2. Aspirate medium from hESCs, and add 1 mL/well of collagenase IV splitting medium into each well of the 6-well plate.

3. After incubation at 37 °C in incubator for 5 min, scrape the hESC colonies from the plate with a 5 mL pipet and transfer cells to a 15 mL sterile falcon tube.

4. Add 1 mL of EB differentiation medium—medium 1 (*see* Subheading 2.1.1, item 15) into each well. Scrape off the remaining cells in the well with a cell scraper, and pool the cells together in the 15 mL falcon tube. Gently pipet the cells up and down a few times in the tube, to further break up the colonies.

5. Allow the cell clumps to settle down for 5–10 min. Aspirate the supernatant, and then resuspend the cells with medium 1 to give a final volume of 3 mL per well of a 6-well ultra-low attachment microplate. The splitting ratio is set at 1:1, where one 6-well plate of hESCs is split to form one 6-well plate of EBs.

6. After overnight culture in suspension, hESCs form floating aggregates known as EBs. Culture medium is changed every second day. To change medium, transfer EBs into 50 mL falcon tube and let the aggregates settle for 5–10 min. Aspirate the supernatant, replace with fresh medium, and transfer back to the 6-well ultra-low attachment microplates for further culture.

7. At day 5, EBs are harvested for induction of chondrogenic differentiation.

3.2 Micromass Culture of EB-Derived Cells

1. Transfer the 5'd' EBs into a sterile 50 mL falcon tube and allow the EBs to settle for 3–5 min. Aspirate the supernatant and wash once with PBS (*see* **Note 8**).

2. 5'd' EBs are then dissociated into single cells by dissociation using a solution mixture containing 0.05 % trypsin/EDTA and cell dissociation buffer at 7:3 ratio for 5 min at 37 °C, followed by passing the cell suspension through a 22-G needle and then a 40-μm cell strainer to obtain single cells suspended in pre-warmed medium 1. The serum-containing medium helps to inactivate the trypsin (*see* **Note 9**).

3. Wash twice with the same medium by spinning down the cells at $250 \times g$ for 5 min and resuspending in medium. At the second spin, resuspend the cells in a lower volume of medium for cell count.

4. Culture cells at a high density of 3×10^5 cells per 15 μL spot in a 24-well plate pre-coated with 0.1 % gelatin (*see* **Note 10**). After incubation for approximately 2 h, 500 μL of the same medium was carefully added to each well. Micromass cultures

were incubated overnight at 37 °C to enable cell attachment, prior to induction of differentiation the next day.

5. Following incubation for 24 h to allow cell attachment, replace with serum-free chondrogenic medium—medium 2 (*see* Subheading 2.1.1, item 16) with TGF-β1 (10 ng/mL) (*see* Subheading 2.1.2, item 1) for a period of 21 days with medium change every alternate day (*see* **Note 11**). Example of micromass cultures cultured in TGF-β1-supplemented chondrogenic medium is shown in Fig. 1c. Cultures in the basic serum-free chondrogenic medium without growth factor supplementation will serve as the control.

3.3 Isolation and Expansion of Chondrogenic Cells

1. Micromass cultures are digested overnight with Collagenase P (1.5 mg/mL) digestion to release the chondrogenic cells.

2. Pass the cells through the 40-μm nylon cell strainer.

3. Only single cells derived after collagenase digestion and passing through the strainer are plated for further expansion.

4. Depending on the cell yield, the cells can be directly used for pellet/hydrogel cultures or expanded to higher numbers on collagen II-coated plates. Medium 3 (*see* Subheading 2.1.1, item 17) supplemented with TGF-β1 (1 ng/mL), FGF-2 (5 ng/mL), and PDGF-bb (10 ng/mL) is used to expand the chondrogenic cells whilst maintaining their differentiation potential. Passage the cells at seeding density of 5×10^4 cells per cm^2. The cells can be expanded up to 5 passages, without notable loss in differentiation potential. The cells adopt a spindle-like morphology upon expansion (*see* Fig. 1d).

5. Freeze the chondrogenic cells at each passage at 2×10^6 viable cells/mL of freezing medium—Medium 3 supplemented with additional 10 % FBS, and 10 % DMSO.

3.4 Pellet Culture System

1. For pellet culture (17, 18), spin down the cells at $300 \times g$ and seed the cells at high density, approximately 2×10^5 cells per 500 μL in 15 mL falcon tubes. Medium 3 (*see* Subheading 2.1.1, item 17) is used to allow cell recovery at Day 0.

2. Pellet formation could be observed the next day. Gently tap the tube to dislodge the pellet from tube bottom (Day 1)

3. At Day 1, perform medium change to differentiation medium—medium 4 (*see* Subheading 2.1.1, item 18) supplemented with various growth factors (*see* Subheading 2.1.2), 500 μL per pellet.

4. Allow the pellets to further differentiate for another 14–28 days with medium change every alternate day. Matrix (s-GAG) synthesis by hESC-derived chondrogenic cells can be analyzed by biochemical assays of s-GAG and DNA, as
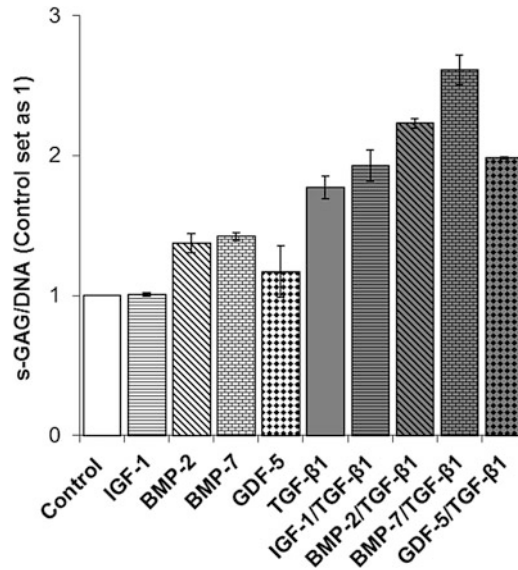
Fig. 2 Growth factor induction of hESC-derived chondrogenic cells. Human ESC-derived chondrogenic cells were cultured as pellets for 14 days in chondrogenic medium (medium 4) supplemented with various growth factors singly or in combination (*see* Subheading 2.1.2). Analysis of s-GAG and DNA was performed. The combination of 100 ng/mL BMP-7 and 10 ng/mL TGF-β1 demonstrated synergistic effects in stimulating maximum chondrogenesis, as observed by the highest level of s-GAG synthesis by day 14 of differentiation over other growth factors

shown in Fig. 2. Culture of hESC-derived chondrogenic cells in chondrogenic medium supplemented with BMP-7 (100 ng/mL) and TGF-β1 (10 ng/mL) resulted in superior s-GAG synthesis.

3.5 3-D Hydrogel Culture System

1. Prepare the HyStem-C hydrogel kit components by adding the required amount of DG water into individual components under aseptic conditions, following the manufacturer's instructions.

2. Mix 1 mL of Glycosil® and 1 mL of Gelin-S®.

3. Prepare the hESC-derived chondrogenic cells. Spin down the cells at $300 \times g$ and concentrate in 100 μL of medium 3 (*see* Subheading 2.1.1, item 17) to the required seeding density. Recommended seeding density is approximately 2×10^6 cells/100 μL construct.

4. Add 0.1 mL cells into the 2 mL mixture of Glycosil® and Gelin-S®. Pipette up and down to mix well.

5. Add 0.5 mL of Extralink® to the mixture of cells and Glycosil® + Gelin-S®. Once the Extralink® is added, the mixture will take less than 20 min to form hydrogel.

6. Pipette up and down to mix well. Pipette 100 μL of the cell-hydrogel mixture into each transwell insert. Do not add medium at this point.

7. Allow complete gelation of the cell-hydrogel construct by incubating the plate in the cell culture incubator at 37 °C and 5 % CO_2 for at least 1 h.

8. Remove the plate. Verify the hydrogel formation using a pipette tip. Add 1.5 mL of medium 3 into each well. Place back into the cell culture incubator.

9. At Day 1, perform medium change to differentiation medium—medium 4 (*see* Subheading 2.1.1, item 18) supplemented with BMP-7 (100 ng/mL) and TGF-β1 (10 ng/mL), 1.5 mL per construct.

10. Allow the constructs to further differentiate for another 14–28 days with medium change every alternate day. At the point of harvest, each construct is cut in half into two pieces for both biochemical and histological analysis. The cross section of the cartilage tissue construct stained by Alcian blue is shown in Fig. 1e.

3.6 Biochemical Analysis

3.6.1 s-GAG Assay

1. Remove the culture medium from the pellet or hydrogel cultures. Remove the constructs from the transwell inserts by using a forceps to separate the transwell membrane. Wash once with PBS. Remove the PBS completely. Transfer the samples (pellet or construct) into sterile 1.5 mL microtubes.

2. Add 500 μL papain digestion buffer to each sample and incubate in water bath at 60 °C for 18–24 h.

3. Sample supernatants can be assayed immediately or frozen down at −20 °C for subsequent assays of s-GAG and DNA.

4. s-GAG content is measured spectrophotometrically at 655 nm using the Blyscan s-GAG assay kit (*see* Subheading 2.2.1, item 2) and normalized to the DNA content, measured fluorometrically using the Picogreen method (*see* Subheading 2.2.1, item 3).

5. Prepare serial dilutions of the standard (50, 25, 12.5, 6.25, 3.13, 1.56 μg/mL) using PBS.

6. Add 50 μL of standards and samples to 250 μL of Blyscan dye reagent in 1.5 mL microtubes and incubate at RT for 30 min.

7. After incubation, centrifuge the sample at $16,000 \times g$ for 10 min. Discard the supernatant, but retain the dye-bound pellet, which is then dissociated with 250 μL dissociation agent (supplied in the assay kit) per sample to release the color.

8. Transfer 200 μL of each sample to individual wells of a 96-well microwell plate for spectrophotometric measurement at 655 nm. The dissociation agent is used as blank.

9. Evaluate the amount of s-GAG per sample from the standard curve. If the sample reading is outside the standard curve range, it is recommended that another dilution and reading of the sample be performed.

3.6.2   DNA Assay

1. Thaw the Picogreen® dye at RT in the dark.

2. Prepare the DNA standard working solution (2 μg/mL). Add 6 μL of DNA stock (100 μg/mL) to 294 μL of TE buffer. Vortex to mix well.

3. Prepare dilutions of the DNA standards, as shown in Table 1.

4. Dilute 10 μL of sample ten times with 1× TE buffer to a final sample volume of 100 μL. Sample dilution can be performed in 1.5 mL microtubes or directly in the 96-well black microplate.

5. Add 100 μL of standards and samples into each well. Vortex the samples before adding.

6. Dilute the Picogreen® dye with 1× TE buffer. For one 96-well plate, prepare sufficient Picogreen® working reagent for 110 wells. Add 55 μL of Picogreen dye to 11 mL of 1× TE buffer solution. Prepare just before use.

7. Dispense 100 μL Picogreen® working reagent to each well using a multichannel pipette.

8. Take fluorescence readings using the plate reader (Safire, Tecan) with excitation at 485 nm and emission at 535 nm. Evaluate the amount of DNA per sample from the standard curve. If the sample reading is outside the standard curve range, it is recommended that another dilution and reading of the sample be performed.

3.7   Histological Analysis

3.7.1   Processing of Cartilage Samples

1. Fix each cartilage pellet or construct in 10 % buffered formalin overnight at 4 °C.

2. Dehydrate the constructs in successive ethanol washes of 70, 70, 80, 80, 95, and 95 %, for 15 min each.

3. Briefly stain the constructs with eosin, followed by two brief rinses in 100 % ethanol. This stain serves to visualize the constructs and aid in their handling.

4. Incubate the constructs in three changes of 100 % ethanol, 20 min each.

5. Transfer to two changes of xylene, 20 min each.

6. Transfer to three changes of melted paraffin, 20 min each.

7. Remove each construct and embed in paraffin block as per standard embedding procedure. Allow the paraffin block to solidify completely.

8. Cut 5 μm sections of each construct and transfer 2–3 sections onto each microscope glass slide.

9. Store the slides at RT for future staining.

3.7.2 Hematoxylin and Eosin Staining

1. Deparaffinize the sections on slides in two changes of xylene, 10 min each.

2. Rehydrate the sections in serial washes of 100 % ethanol, 95 % ethanol, and 70 % ethanol, 2 min each.

3. Hydrate the sections in dH_2O for 5 min.

4. Stain the sections with hematoxylin for 5 min, and then rinse thoroughly in several changes of tap water.

5. Dip the sections briefly in differentiating solution (*see* Subheading 2.3, item 7), then rinse in tap water.

6. Blue the sections in Scott's tap water, and then wash in two changes of tap water.

7. Stain with eosin for 2 min. Rinse briefly in two changes of 95 % ethanol followed by two changes of 100 % ethanol.

8. Clear the sections in three changes of xylene, 5 min each.

9. Air-dry for 5 min and coverslip with xylene-based DePex mounting medium.

10. View by light microscopy (*see* Fig. 3).
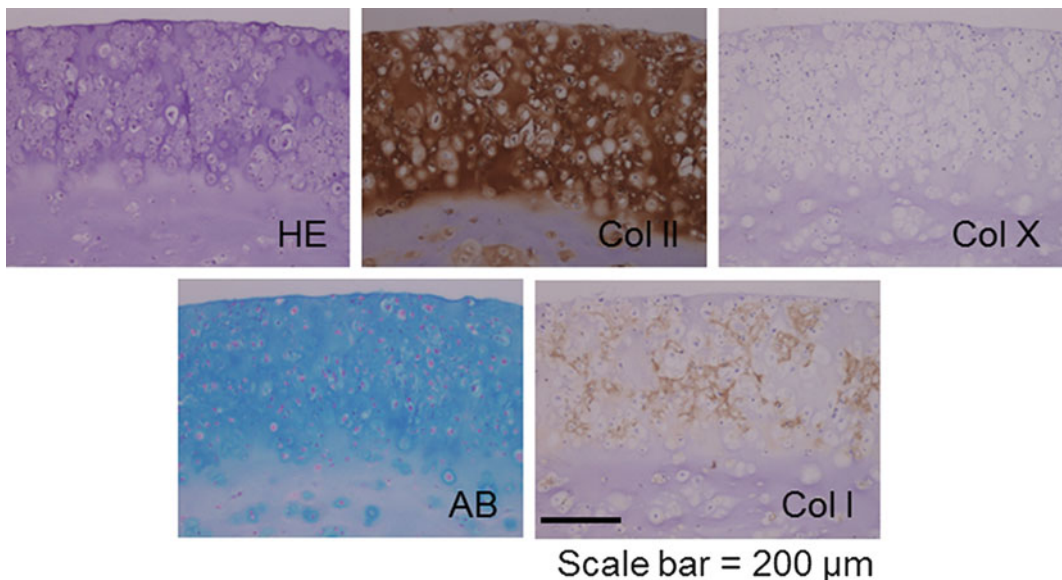
Fig. 3 Histological assessment of cartilage tissue construct based on hESC-derived chondrogenic cells cultured in HA-based hydrogel. Hematoxylin and Eosin (HE), Alcian blue (AB), and immunohistochemical staining specific for collagen type II (Col II), type I (Col I), and type X (Col X) collagens were performed. Human ESC-derived chondrogenic cells cultured in HA-based hydrogel in the presence of 100 ng/mL BMP-7 and 10 ng/mL TGF-β1 formed hyaline cartilage by the end of 28-day culture, with high amounts of s-GAG and collagen II, and low amounts of collagen I and X

3.7.3 Alcian Blue Staining

1. Deparaffinize the sections on slides in two changes of xylene, 10 min each.

2. Rehydrate the sections in serial washes of 100 % ethanol, 95 % ethanol, and 70 % ethanol, 2 min each.

3. Hydrate the sections in dH_2O for 5 min.

4. Stain the sections with Alcian blue solution for 30 min (*see* Subheading 2.3, item 3).

5. Rinse in tap water.

6. Counterstain with nuclear fast red for 5 min.

7. Rinse in tap water.

8. Dehydrate in successive ethanol washes of 70, 95, and 100 % ethanol, 3 min each.

9. Clear the sections in three changes of xylene, 5 min each.

10. Air-dry for 5 min and coverslip with xylene-based DePex mounting medium.

11. View by light microscopy (*see* Fig. 3).

3.7.4 Collagen I, II, X Immunohistochemistry

1. Deparaffinize the sections in two changes of xylene, 10 min each.

2. Rehydrate the sections in serial washes of 100 % ethanol, 95 % ethanol, and 70 % ethanol, 2 min each.

3. Hydrate the sections in PBS for 5 min.

4. Circle the sections with a wax pen.

5. To facilitate antigen retrieval and antibody access, incubate with pepsin at 37 °C for 20 min.

6. Rinse once with PBS, then block with hydrogen peroxide block for 15 min at RT to quench any endogenous peroxidase activity.

7. Wash four times with PBS, block with Ultra V Block prior to 1 h incubation at RT with primary antibodies (*see* Table 2).

Table 2
List of primary antibodies used in immunohistochemistry

Antibodies	Host	Dilution	Incubation time (min)	Source
Collagen I	Mouse	1:1,000	60	Sigma
Collagen II	Mouse	1:500	60	Chemicon
Collagen X	Mouse	1:25	60	Quartett
IgG_1 control	Mouse	–	60	Invitrogen

At the end of primary antibody incubation, wash four times with PBS, before adding the pre-diluted biotin-conjugated goat-derived anti-mouse secondary antibody and incubate at RT for 30 min (*see* Subheading 2.4, item 3).

8. At the end of secondary antibody incubation, wash four times with PBS, before incubating with streptavidin-conjugated horseradish peroxidase at RT for 45 min.

9. Wash four times with PBS before adding the DAB chromogen to visualize the antibody-antigen reaction.

10. Counterstain with Mayer's hematoxylin for 2–3 min, then wash in tap water.

11. Blue in Scott's tap water before rinsing in tap water.

12. Dehydrate in successive ethanol washes of 70, 95, and 100 % ethanol, 3 min each.

13. Clear the sections in three changes of xylene, 5 min each.

14. Air-dry for 5 min and coverslip with xylene-based DePex mounting medium.

15. View by light microscopy (*see* Fig. 3).

4   Notes

1. Stock preparation of dexamethasone is stable for 6 months at $-20\ ^\circ C$ in a non-defrosting freezer.

2. 1 % KSR instead of a strictly serum-free medium was used in this study, based on reports that mesodermal differentiation of ESCs may be inhibited under serum-free conditions (19).

3. Use chondrogenic differentiation medium for not more than 2 weeks after preparation, as components such as L-glutamine and AA2P degrade over time. Store the chondrogenic differentiation medium at 4 $^\circ$C and discard unused medium after 2 weeks.

4. Avoid repeated freeze-thaw cycles of the growth factors. Once thawed, keep the growth factor aliquot at 4 $^\circ$C and use within 1 week.

5. DAB is a potential carcinogen and needs to be handled with care and disposed according to the laboratory safety regulations.

6. Hydrogels are the most common biomaterials for injectable cartilage tissue engineering and can be derived from natural and synthetic polymers (20, 21). Several advances have been made in recent years in the hydrogel design and engineering for regenerative medicine applications (22, 23).

7. Human ESC cultures [H1 and H9] follow exactly as recommended by the Wicell protocol. Refer to website http://www.wicell.org/ for protocols describing the expansion and propagation of hESCs on MEF feeder cells.

8. Allow the EBs to settle down during the wash with PBS. Usually it takes less than 3 min for all the EBs to settle. Avoid pipetting too many times or foaming because the cells should be collected in clumps.

9. Dissociate the EBs into single cells for no more than 5 min and pass the cell suspension once through a 22-G needle and then 40-μm cell strainer. Prolonged or repeated dissociation is detrimental to cell viability.

10. The method for micromass culture has been modified from previously published methods (24, 25). After removal of 0.1 % gelatin from the wells, ensure the plates are completely dry before use. This is to prevent dispersion of cells when dispensing the cells in a single drop onto the well in a micromass culture.

11. Efficiency of chondrogenic differentiation and commitment of hESCs to chondrogenic lineage is enhanced with growth factor stimulation, especially TGF-β1. However, it is important to note that the effect of TGF-β1 on chondrogenesis is stage-dependent where TGF-β1 exerts early inhibitory effect on hESCs and only promotes chondrogenesis after EB formation (26).

Acknowledgements

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Microgrooved Surface Modulates Neuron Differentiation in Human Embryonic Stem Cells

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Abstract

Stem cell-based therapies have drawn intensive attention in the neuronal regenerative fields. Several studies have revealed that stem cells can serve as an inexhaustible source for neurons for transplantation therapies. However, generation of neurons and directionality has not yet been fully investigated. Herein, we investigate the mechanical ramifications of surface topography on human embryonic cell differentiation. Microgrooved surfaces with various pitches were applied to modulate the neuron differentiation. Our protocol showed that neuron differentiation increased as grove pitch decreased. The results indicated that 2 μm microgrooves can improve neuron growth by ~1.7-fold. Our results indicate the importance of mechanotransduction on neuronal differentiation and highlight the feasibility of manipulating the neuronal differentiation with surface topography, providing new perspectives for accommodating clinical transplantation.

Keywords: Stem cell, hESC, Neuron, Differentiation, Microgrooves, PDMS

1 Introduction

Neuron-related diseases and injury are an increasingly pressing issue in our society (1, 2). Currently, there are more than 2.5 million people suffering from spinal cord injuries (SCI) worldwide (1). Furthermore, the increasing patient population of neurological disorders, such as Parkinson's disease, has caught the publics' attention (3, 4). Due to the low regenerative capacity of neuronal cells, various molecular and cellular therapies have been conducted in an attempt to help bridge the injury sites and promote the neuronal conductions (1, 2). For instance, molecular therapies, such as brain-derived neurotrophic factors (BDNF) and nerve growth factor (NGF), have been utilized to induce axon growth (1, 5). Recently, with the developments of stem cell-based approaches, stem cell therapy provides the advantages of both cellular and molecular therapies; additional investigations have addressed these potential applications of stem cell therapies for neuro-regenerative medicine (2, 6).
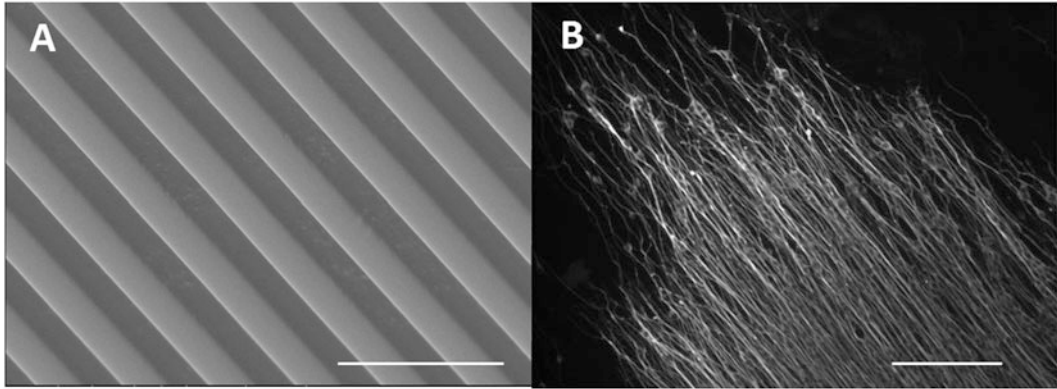
Fig. 1 Effect of surface topography on orientation of neurons differentiated from hESCs. SEM images of (**a**) microgroove; Scale bar: 10 μm. Fluorescence images of neuron alignment (**b**) regulated by surfaces (**a**). Neuron axons were indentified with β-tubulin; Scale bar: 200 μm
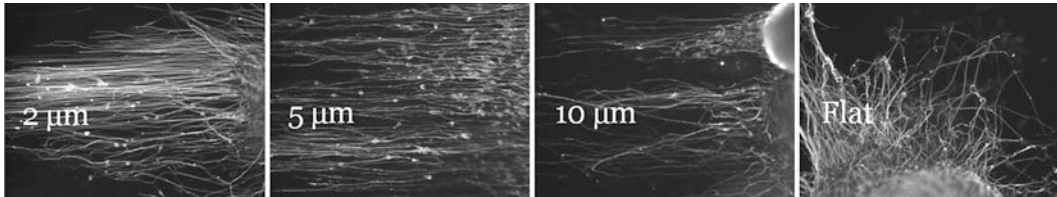
Fig. 2 Human embryonic stem cells (hESCs) derived neuronal progenitor with varying microgroove pitches. Immunofluorescence of β-III tubulin of neurons differentiated on 2 μm, 5 μm, 10 μm microgroove pitches and a flat surface

The unique self-renewal and pluripotent properties of human embryonic stem cells (hESCs) have been specifically targeted for potential treatments in neuron regenerative medicine (6–8). To enhance the efficacy of stem cell therapies, various protocols have been developed for guiding hESCs to differentiate into various neuronal lineages (2). The impacts of physical microenvironments, such as surface topography and substrate elasticity, on stem cell fates have been investigated (9, 10). For example, synthetic nanofiber matrixes have been designed to promote rapid neuron differentiation (11), and different types of differentiated neuronal cells were found on nanofibers of different diameters (12, 13).

Herein, we demonstrate that the physical features can be utilized to guide specific directional cell growth with microgrooves (Fig. 1). Additionally, the surface microgrooves can also be applied to physically modulate the specific differentiation of neurons from hESCs. Our results have shown that the neurons grown on the microgrooves are uniform in direction and increased in neuronal differentiation as the microgroove pitches decreased (Fig. 2). 2 μm microgrooves have shown the best results in neuron differentiation, ~1.7-fold than neuron differentiation on regular flat surfaces, and

directionality. Our method of microgrooved differentiated hESC into neurons could contribute to neuronal cell therapies and the understanding of mechanotransduction impacts on stem cell fate determination.

2   Materials

2.1   Polydimethyl-siloxane (PDMS)

SYLGARD 184 SILICONE ELASTOMER KIT (Dow Corning).

1. SYLGARD 184 Silicone Elastomer Base.
2. SYLGARD 184 Silicone Elastomer Curing Agent.
3. Soft lithography.

2.2   Feeder Cell Components

1. Mouse embryonic fibroblasts (MEFs, Millipore).
2. MEFs medium: Dulbecco's modified Eagle's medium (DMEM/F12; Invitrogen), 20 % Fetal Bovine Serum (Invitrogen), 1 mM L-glutamine (Invitrogen), 1 % nonessential amino acids (Invitrogen). Filter solution with 0.22 μm filter into a bottle and store at 4° C. Should make fresh every 2 weeks.
3. Dissolve 2 mg Mitomycin C (Sigma-Aldrich) in 200 ml of MEFs medium. Mitomycin C is light sensitive so please avoid direct light.
4. Tissue culture plates.

2.3   Human Embryonic Stem Cell Components

1. Human embryonic stem cells (hESCs): (H9, WiCell, Madison, WI).
2. hESCs medium: 20 % KO serum replacement (Invitrogen), 1 % nonessential amino acids (Invitrogen), 1 mM L-glutamine (Invitrogen), Dulbecco's modified Eagle's medium (DMEM/F12; Invitrogen), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), and 4 ng/ml FGF-2 (Sigma-Aldrich). Filter solution with 0.22 μm filter into a bottle and store at 4° C. Should make fresh every 2 weeks.
3. Dissolve 1 mg/ml Dispase (Invitrogen, CA) into Dulbecco's modified Eagle's medium (DMEM/F12; Invitrogen).
4. Embryonic Bodies (EBs) medium: hESCs Medium without FGF-2.
5. Neuron induction medium: Dulbecco's modified Eagle's medium (DMEM/F12; Invitrogen), 1 % nonessential amino acids (Invitrogen), 1 % Sodium Pyruvate (Invitrogen), 1 mM L-glutamine (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), and 4 ng/ml FGF-2 (Sigma-Aldrich), 1 % N2 supplement (Invitrogen), and FGF2 (20 ng/ml, Invitrogen).
6. Ultra-low attachment dish: (Costar, Fisher).
7. Laminin (Sigma-Aldrich).

2.4 Immunohisto-
chemical Staining

1. Phosphate buffer saline (PBS; Fisher).

2. 4 % Paraformaldehyde: 2 g paraformaldehyde in 100 ml PBS. Heat mixture to 55° C on a hot plate with a stir bar to incorporate the mixture. Add 4–5 drops of 10 N NaOH and allow the mixture to clear up. Neutralizes the pH to 7.3 with HCl and filter solution with 0.22 µm filter to remove particulates. Should make fresh every 2 weeks. CAUTION: NaOH and HCl are highly corrosive so use safety precaution while using these chemical. Paraformaldehyde is a fixative, so use safety precaution while handling these chemical.

3. 0.1 % Triton X-100 (Sigma-Aldrich) in PBS.

4. 2 % Bovine serum albumin (BSA) in PBS.

5. PBST: PBS with 0.05 % Tween 20.

6. Primary antibodies: Anti-β-tubulin III (1:500, Millipore), Anti-Olig2 fluorescent phalloidin (A12379, Invitrogen), Focal adhesion kinase (FAK, Millipore), vinculin (Millipore).

7. Secondary antibodies: Alexa Fluor 488 (1:500, Invitrogen) and Alexa Fluor 555 (1:500, Invitrogen).

3   Methods

3.1   PDMS

To prepare desired amount of PDMS mixture.

1. Microstructure molds (microgroove with 3 µm height and 2, 5, or 10 µm pitches, respectively) are fabricated on silicon wafers with SU-2050 by standard photolithographic techniques.

2. Combine SYLGARD 184 Silicone Elastomer Base and SYLGARD 184 Silicone Elastomer Curing Agent in a 10:1 ratio then mix thoroughly (*see* **Notes 1** and **2**).

3. Cast mixture into desired mold and adjust thickness to specific application.

4. Place mold onto platform rocker and rock at intermediate-high speed for 45 min.

5. Place molds into 37 °C incubator overnight to allow consolidation (*see* **Note 3**).

3.2   Human
Embryonic Stem
Cell Culture

Cell culture and treatments are incubated at 37° C. Highly recommend that all cell work should be done in a biosafety hood to prevent contamination and use proper sterilization techniques.

1. Amplify and culture MEFs cells with MEFs medium. Then Mitomycin C treat the MEFs for 2 h (*see* **Note 4**).

2. Replate and seed MEFs at 12,000 cells/cm^2. Allow cells to attach in the incubator overnight.

3. Wash MEFs plate with PBS to remove residual MEFs medium and aspirate the solution.

4. Plate H9 hESCs line with hESCs medium on MEFs plate. Change medium everyday to ensure a healthy culture and minimal differentiation.

5. Subculture hESCs every 7 days on to new MEFs plates to maintain culture by manually cutting and selecting healthy and undifferentiated colonies to be subcultured (*see* **Note 5**).

6. Generation of Embryonic Bodies (EBs): Incubate Dispase with the hESCs for 10–15 min to allow colonies to detach from the MEFs feeder layer (*see* **Note 6**).

7. Plate hESC colonies into the ultra-low attachment plate and culture for 5–7 days with EBs medium. Colonies should form into a sphere-like shape called an embryoid body (*see* **Note 7**).

3.3 Microgroove Stem Cell Differentiation

1. Once desired microgroove is formed and sterilized with 70 % ethanol, then coat PDMS in 100 μg/ml laminin solution overnight.

2. Wash PDMS with PBS and seed EBs on to PDMS substrate with Neuron induction medium. Incubate for 7 days while changing medium every day (*see* **Note 8**).

3.4 Immunohisto-chemical Staining

All incubation time was done at room temperature.

1. Once the hESCs are done incubating on the PDMS substrate for 7 days. Wash with PBS and fix cells with 4 % paraformaldehyde for 35 min at room temperature and wash with PBS.

2. Treat cells to 0.1 % Triton X-100 for 10 min and wash with PBST.

3. Cells were then blocked with 2 % BSA for 1 h and then washed with PBST.

4. Cells were then incubated with primary antibodies for 1 h then washed with PBST 3 times (*see* **Note 9**).

5. Incubate cells with secondary antibodies for 1 h and wash cells with PBST 3 times.

6. Store samples in PBS and cells are ready for imaging.

4 Notes

1. If mixture is too viscous, reduce amount of SYLARD 184 Silicone Elastomer Curing Agent.

2. Place combined mixture in vacuum hood until all air bubbles within mixture removed—approximately 1–2 h depending on concentration of air bubbles within mixture.

3. Once in incubator, mold can last for 2 weeks but will not exhibit changes beyond that point. Storing prepared mold longer than 2 weeks, though, is not recommended.

4. Make a large batch of MEFs so that you can freeze the cells after Mitomycin C treatment to have cells ready when needed. Mitomycin C is light sensitive so when making the solution, avoid light and wrap bottle in foil to store.

5. Shake plate well to disperse new colonies to allow ample growth and minimize differentiation.

6. Check plate at 10 min to see if the hESC colonies are lifting off by the edge of the colonies. Colonies should be easy to remove by pipetting solution over the colonies, careful not to shred the colonies while pipetting. Beware of incubating with dispase for too long, because MEFs layer will detach from the plate and cause difficulty when trying to remove only the hESCs colonies.

7. Separate the colonies when they are forming EBs, EBs will fuse together if the EBs are in close proximity.

8. Seed EBS apart from each other to allow maximum growth of axons in the microgrooves.

9. When washing cells with PBST, for best result place cells on a shaker to slowly agitate the solution. This will help remove nonspecific staining.

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Directed Differentiation of Human Embryonic Stem Cells into Neural Progenitors

Erin Banda and Laura Grabel

Abstract

A variety of protocols have been used to produce neural progenitors from human embryonic stem cells. We have focused on a monolayer culture approach that generates neural rosettes. To initiate differentiation, cells are plated in a serum-free nutrient-poor medium in the presence of a BMP inhibitor. Depending on the cell line used, additional growth factor inhibitors may be required to promote neural differentiation. Long-term culture and addition of the Notch inhibitor DAPT can promote terminal neuronal differentiation. Extent of differentiation is monitored using immunocytochemistry for cell type-specific markers.

Keywords: Human embryonic stem cells, Neural progenitors, BMP, Notch

1   Introduction

There is tremendous interest in simple protocols designed to promote the neural differentiation of human pluripotent stem cells, since this material can be used for cell replacement therapies, in vitro disease modeling, and drug testing. Over the years many approaches have been taken to promote neural differentiation, and most rely, at least in part, upon the concept that neural differentiation occurs largely through inhibition of major signaling pathways (1). This concept can be traced back to the discovery of the primary organizer, a region of the early embryo that can induce a secondary dorsal axis (2). When the active molecular components localized to this region were identified, they included inhibitors of both the BMP and Wnt pathways (3). Early neural differentiation is therefore promoted in nutrient-poor medium that is devoid of growth factors by the so-called default mechanism (4). Since the differentiating embryonic stem cells (ESCs) may condition the medium with growth factors, for some cell lines it may be necessary to add inhibitors of the BMP or the Wnt pathways to promote neural differentiation (5, 6).

Despite distinct growth characteristics for mouse versus human ESCs, protocols designed for neural differentiation tend to work well with cells from either species, and for induced pluripotent stem

cells (iPSCs) as well, with no or little modification. Neural differentiation protocols fit broadly into three major categories: embryoid body intermediate, monolayer co-culture with another cell type, or monolayer culture alone (7). ESCs placed in suspension culture form aggregates capable of producing the three primary germ layers: ectoderm, mesoderm, and endoderm. Subsequent plating of these embryoid bodies on adhesive substrates, under serum-free, growth factor-depleted conditions, promotes neuroectoderm differentiation. Embryoid body protocols have been modified to produce abundant ectoderm derivatives (8), but a concern remains that this approach includes the initial production of multiple germ layer lineages, which must subsequently be winnowed down to neuroectoderm–ectoderm alone, usually by culture in a nutrient-poor medium. Co-culture with bone marrow stromal cells, or conditioned medium derived from these cells, greatly enriches for neural derivatives in monolayer culture (9).

We have focused on a monolayer protocol in which human ESCs are plated directly into a nutrient-poor medium onto substrates coated with poly-L-lysine plus laminin. During differentiation the cells are passaged mechanically, as described below, though once neural stem cells are present enzymatic passage may be used (Fig. 1, see Section 3.1.5). To optimize for neural differentiation of human ESCs, a BMP inhibitor must be added as described below. We have used both noggin and the small-molecule inhibitor LDN. Whereas noggin is a broad-spectrum BMP inhibitor acting outside of the cell, LDN targets BMP type I receptor kinases, inhibiting phosphorylation of Smads 1, 5, and 8 (10). Some reports suggest that noggin and LDN could have distinct effects on neural induction, and care should be taken that the desired neural progenitors can be produced. Addition of the Activin/Nodal antagonist SB431542 along with a BMP antagonist, the dual-inhibition protocol, can also be effective at neural induction (11). Inhibition of Wnt signaling may also promote human ESC neural differentiation, and the Wnt inhibitor DKK is most frequently used (12), though we have found its addition unnecessary. It should be noted that the precise composition of the neural induction conditions may vary with specific cell line used.

Under the monolayer conditions we describe here, the differentiating neural progenitors form neural rosettes in which the individual cells elongate and arrange themselves radially (Fig. 2) (13). These rosette structures display many of the properties observed in the developing neural tube and neocortex, including expression of radial glia-specific markers (Fig. 1b), formation of adherens junctions adjacent to a lumen (Fig. 1c), interkinetic nuclear migration (Fig. 1c), and neuronal differentiation at the outer edge away from the lumen (Fig. 1d). The outer rosette edge is characterized by the presence of the extracellular matrix
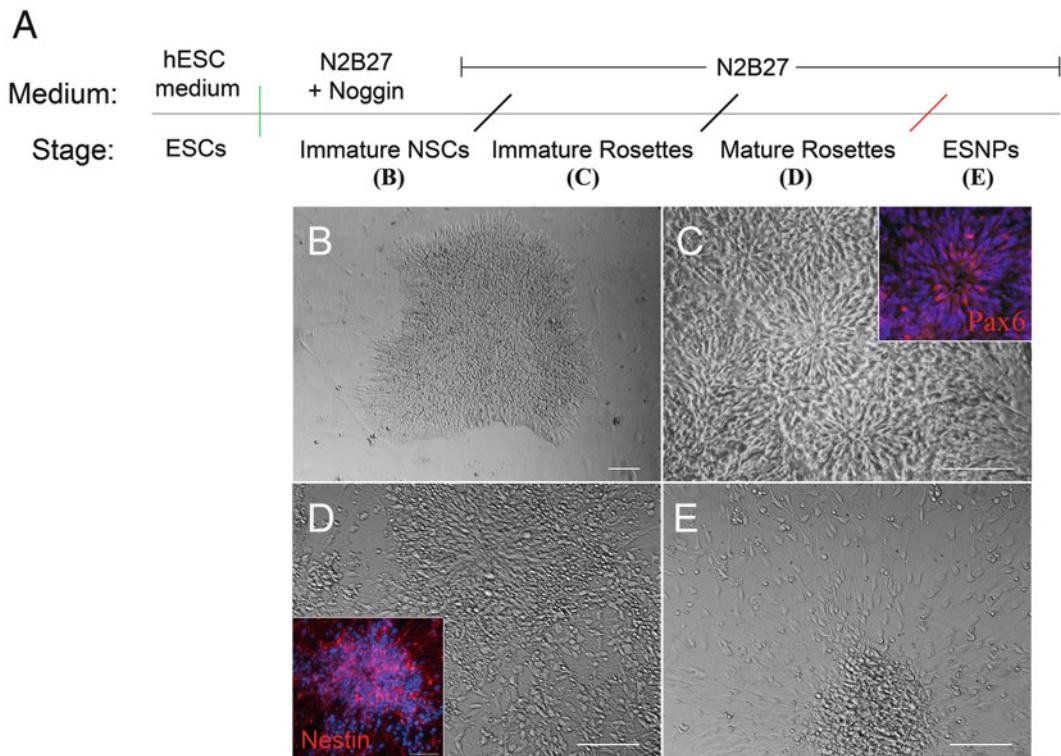
Fig. 1 Protocol for the generation of human embryonic stem cell-derived neural stem cells. (**a**) Schematic for neural differentiation. ESC medium is removed from hESC cultures, replaced with N2B27, and supplemented with the BMP inhibitor noggin (*green line*). Cells are cultured in N2B27+ noggin for approximately 10 days. Once the culture has reached the immature NSC stage, at which point most of the NSCs express the early neural transcription factor Pax6 (**b**), cultures are manually passaged onto poly-L-lysine- and laminin-coated substrates with the StemPro EZ Passage tool (Life Technologies) (*black line*). Post-passage, NSCs are cultured in N2B27, without supplement, until they reach the immature rosette stage, approximately D16, and begin to express the neural stem cell marker nestin (**c**). At this stage, cultures can again be manually passaged (*black line*) at high densities onto poly-L-lysine- and laminin-coated substrates for the formation of mature rosette structures, approximately 5–10 days post-passage (D21–26) (**d**). Mature rosette cultures can be enzymatically passaged for the formation of embryonic stem cell-derived neural progenitors (ESNPs) (**e**)

receptor 1 integrin (Fig. 2e). At day 16, over 90 % of the cells in these rosette cultures are neural stem cells, based upon the expression of the neural stem cell markers musashi 1 (Msi1) and Pax 6. Very few, if any, cells express markers of undifferentiated ESCs, endoderm, or mesoderm (Fig. 2f). In addition, under 10 % of the cells express mature neuronal markers (Fig. 2f). These data demonstrate that this protocol robustly and specifically generates neural progenitors.

Neural progenitors derived from ESCs may not reflect a generic, all-purpose state and may already be specified towards a specific neuronal lineage. Whereas human ESC-derived neural stem cells are biased towards anterior dorsal lineages, addition of appropriate signaling molecules can redirect patterning (8). For example
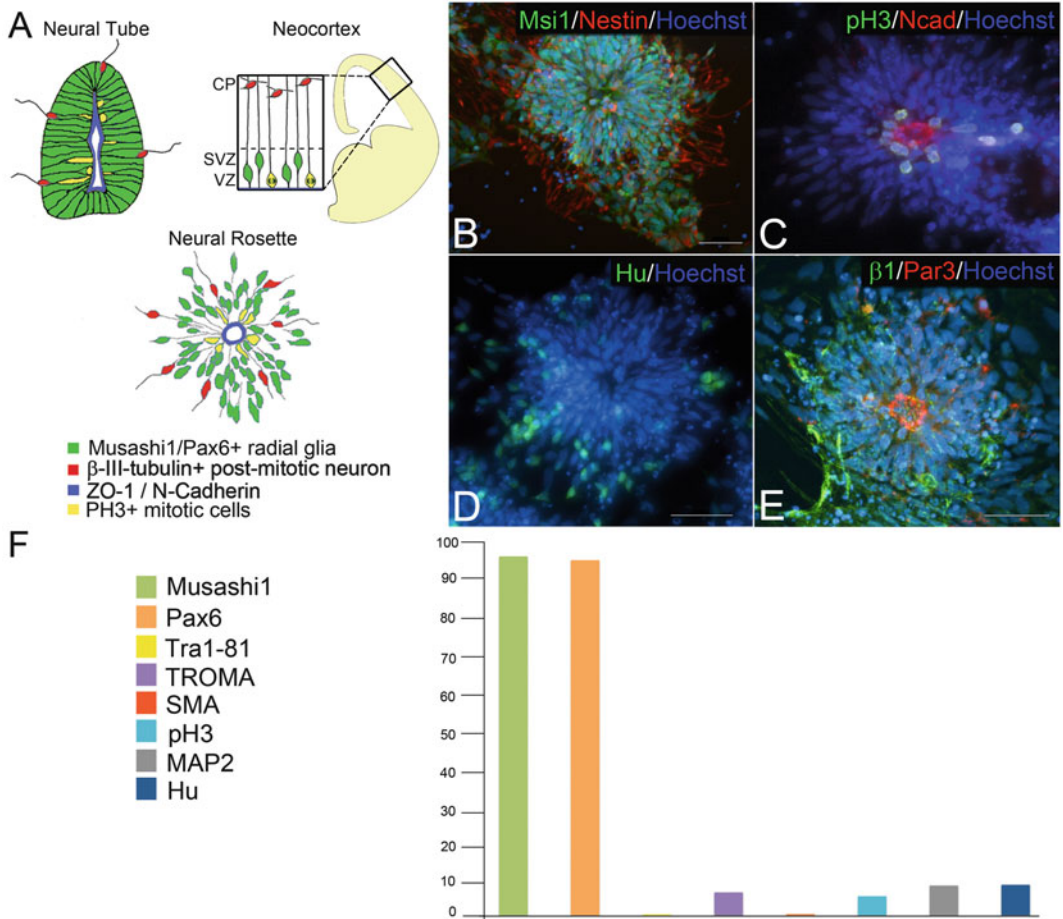
Fig. 2 ESC-derived neural rosettes in vitro bear striking resemblance to the neural tube and developing neocortex. As previously observed, apicobasal polarity is in part established by the restriction of tight junction and adherens junction proteins, ZO1 and N-cadherin (*blue*), to the lumenal/ventricular surface. Neural differentiation within the structures exists as a gradient, with neural stem cells (*green*) closest to the ventricular surface and mature neurons (*red*) at the basal surface (**a**). Rosette NSCs express the neural progenitor markers musashi 1 (Msi1, *green*) and nestin (*red*) (**b**). pHH3+ mitotic cells (*green*) are present in rosettes near the apical surface (N-cadherin, *red*) (**c**). Hu + mature neurons (*green*) appear at the periphery of mature rosettes (**d**). Mature rosette structures form a basal surface indicated by the localization of β1-integrin at the periphery (**e**). Nuclei are labeled by Hoechst in blue. ESC-derived NSC cultures at the immature rosette stage are largely musashi 1 and Pax6 positive, with very few endodermal (TROMA), mesodermal (SMA), or embryonic (Tra1-81) derived cell types. Less than 10 % of the cells in the cultures are neuronal (**f**)

we, and others, have demonstrated that the morphogen Sonic Hedgehog (Shh) can ventralize ESC-derived neural progenitors (8, 13), and addition of Wnt3a directs the formation of dorsal progenitors (8). Using the appropriate signals can redirect ESC-derived progenitors to a variety of specific neuronal subtypes. Differentiation to a wide variety of mature neurons, including dopaminergic midbrain neurons (14), motor neurons (15), and

inhibitory interneurons ([13], [16], [17]), has been documented both in vitro and in vivo. Based upon the observation that Notch signaling promotes maintenance of the neural stem cell state, maturation of ESC-derived neural progenitors to neuronal subtypes can be facilitated by the addition of the Notch inhibitor DAPT ([18]). Terminal differentiation medium (TDM), containing cyclic AMP, ascorbic acid, glial cell line-derived neurotrophic factor (GDNF), and brain-derived neurotrophic factor (BDNF) may also be used to promote neuronal differentiation.

2   Materials

2.1   Cell Culture

Mouse embryonic fibroblast (MEF) cell culture

1. Dulbecco's Modified Eagle's Medium (DMEM) (Sigma), supplemented with 10 % fetal bovine serum (FBS) (Atlas, Fort Collins, Colorado), 1 % L-Glutamine (Life Technologies), and 1 % penicillin/streptomycin (Life Technologies).

2. 0.1 % Porcine gelatin, cell culture grade (Sigma).

Embryonic stem cell culture

1. Dulbecco's Modified Eagle's Medium/F-12 (DMEM/F-12) (Sigma), supplemented with 20 % KnockOut Serum Replacement (KSR) (Life Technologies), 0.5 % L-Glutamine (Life Technologies), 0.4 % penicillin/streptomycin (Life Technologies), 8 ng/ml basic Fibroblast Growth Factor (bFGF) (R&D Systems), 0.1 µM/ml 2-Mercaptoethanol (Sigma).

2. 60 mm cell culture dishes (Corning) coated with 0.1 % porcine gelatin and 2,000 MEFs/cm^3.

3. 18 gauge sterile needle.

4. 1 ml sterile syringe.

5. Dissecting microscope.

Neural Stem Cell Culture (N2B27)

1. Neurobasal Medium (NBM) (Life Technologies), 2 % B27 (Life Technologies), 1 % N$_2$ (Life Technologies), 1 % L-Glutamine (Life Technologies), 1 % Insulin–Transferrin–Selenium (Life Technologies), 1 % penicillin/streptomycin (Life Technologies).

2. LDN193180 (Stemgent).

Poly-L-lysine

1. Boric Acid (Sigma).

2. Sodium Tetraborate (Sigma).

3. Poly-L-lysine (Sigma).

Neuronal differentiation and regionalization

1. Y27632 ROCK-inhibitor (Tocris Bioscience).

2. PBS.

3. Tryple Express (Life Technologies).

4. Quench Medium—10 % FBS (Atlas), NBM.

5. TDM–NBM, 2 % B27, 1 % NEAA, 1 % L-Glutamine, 0.5 μl of 100 mM stock cyclic AMP (cAMP), 50 μl of 200 mM stock ascorbic acid, 50 μl of 10 μg/ml stock GDNF (R&D Systems), 50 μl of 10 μg/ml stock BDNF (R&D Systems).

6. Poly-L-lysine (Sigma).

7. Laminin (Sigma).

8. 8-well glass chamber slides (Nunc Lab-Tek II, Thermo Scientific).

2.2 Immunocyto-chemistry

Fixation

1. Phosphate Buffered Saline (PBS), 3.7 % Formaldehyde (from 37 % stock) (Sigma).

Permeabilization

1. PBS, 0.5 % Triton-X (Sigma).

Immunocytochemistry

1. Blocking buffer: PBS, 0.1 % Triton-X, 10 % Normal Goat Serum (NGS) (Sigma).

2. Wash buffer: PBS, 0.1 % Triton-X.

3. Primary antibodies: Musashi 1 (Abcam), Pax6 (DSHB), Nestin (Chemicon), MAP2 (Sigma), Hu (Sigma), β-III Tubulin (Covance), N-Cadherin (Sigma), Par3 (Cell Signaling), Phospho-histone H3 (Millipore).

4. Alexa-fluor secondary antibodies (Life Technologies): Goat anti-mouse 488, 568, goat anti-rabbit 488, 568.

5. Hoechst-3342 (Life Technologies).

6. Gelvatol with NPG or preferred mounting medium.

3 Methods

3.1 Cell Culture

3.1.1 Preparing MEF Feeder Cell Dishes from Frozen Stocks

1. Coat appropriate number of 6 cm tissue culture dishes with 2 ml of gelatin per dish, making sure to evenly coat the entire bottom of the dish.

2. Allow gelatin to coat dishes for 15 min.

3. Aspirate gelatin off the bottom of the dishes.

4. Add 5 ml of MEF medium to each dish.

5. Thaw vial of mitomycin-C-treated MEFs quickly in 37°C water bath.

6. Take contents of vial, and add to 5 ml of MEF medium in a 15 ml conical tube.

7. Pellet MEFs in centrifuge at $4,000 \times g$ for 3 min.

8. Resuspend MEFs, and plate cells at 5×10^6 per 6 cm tissue culture dish.

3.1.2 Passaging Human Embryonic Stem Cells

1. Aspirate off MEF medium from plated MEF dish and replace with 5 ml of hESC medium.

2. Attach 18 gauge needle to 1 ml syringe.

3. Working under the microscope, use the needle to score colonies into even, square pieces.

4. Using a P200 pipette with tip, gently nudge the scored colony pieces off of the dish while pulling them into the tip. Continue pulling pieces and medium into the tip until full (200 µl), and then add the 200 µl to the new dish.

5. Repeat **step 4**. Add a total of 400 µl of colony pieces to the new dish (this is the equivalent of 10–12 scored colonies transferred).

6. Place in incubator and slide dish gently side to side to ensure proper and even dispersal of colonies over the dish.

7. hESCs typically require passage every 4–6 days. The day of passage should be counted as day 0. Do not change medium on day 1, but beginning day 2, medium should be fully exchanged daily.

3.1.3 Neural Differentiation

1. On day 2 following hESC passage, aspirate off hESC medium and replace with 5 ml N2B27 and supplement with LDN to a final concentration of 100 nM. Noggin may be substituted.

2. Change the medium and supplement with LDN every 48 h through D10 (*see* **Note 1**).

3. On D12, aspirate the medium, replace with N2B27, and continue culture until cells reach the desired endpoint (*see* **Note 2**).

3.1.4 Poly-L-Lysine

1. Add 100 ml of ddH$_2$O to a small beaker with stir bar.

2. Measure 1.25 g of boric acid and add to beaker with water.

3. Begin stirring solution at low–medium speed.

4. Measure 1.91 g of sodium tetraborate and add to beaker.

5. Stir until dissolved.

6. Add 10 mg of poly-L-lysine. Stir and dissolve.

7. Filter and store at −20 until ready for use.

3.1.5 Neuronal Differentiation

1. Coat dishes overnight with poly-L-lysine at RT (*see* **Note 3**).

2. Generously rinse dishes three times with PBS.

3. Coat dishes with laminin at a concentration of 5 μg/ml in PBS. Leave overnight in a 37°C incubator.

4. On D18 of differentiation, treat cells with 10 μM (final concentration) Y27632 for 1 h (*see* **Note 4**).

5. Rinse cells with PBS, and add 2 ml of Tryple Express to the dish.

6. Place dish into a 37°C incubator for 5 min.

7. Remove dish from incubator, and add 2 ml of quench medium.

8. Using a pipette, draw up the Tryple Express + quench medium and rinse the medium over the surface of the dish to encourage cells into suspension (*see* **Note 5**).

9. Add cells and medium to a 15 ml conical tube and spin down at 4,000 × *g* for 3 min.

10. Remove supernatant and resuspend in appropriate volume of N2B27.

11. Remove laminin in PBS from wells.

12. Add 2.5×10^5 cells per well of the 8-well chamber slide ($2.5 \times 10^5/0.8$ cm^2).

13. Final well volume should be 400 μl supplemented with Y27632 at a final concentration of 10 μM.

14. Aspirate off the medium 48 h post-passage and replace with 400 μl TDM.

15. Continue medium changes every other day or as needed until cells reach the desired maturity (*see* **Note 6**).

3.2 Immunocytochemistry

1. Wash cells three times for 5 min with PBS at room temperature (RT).

2. Remove PBS, and add 3.7 % formaldehyde. Fix for 10 min.

3. Wash cells three times for 5 min with PBS.

4. Remove PBS, and add 0.5 % Triton-X in PBS. Permeabilize for 12 min.

5. Remove 0.5 % Triton-X/PBS, and add blocking buffer to cells for 1 h at RT.

6. Remove blocking buffer and incubate in primary antibody overnight at 4°C. Dilute primary antibody in blocking buffer. Antibody concentrations are as follows:

    (a) Rabbit anti-musashi 1 at a 1:250 dilution for neural stem cells.

    (b) Mouse anti-Pax6 at a 1:40 dilution for neural stem cells.

   (c) Mouse anti-nestin at a 1:200 dilution for intermediate filaments of neural stem cells.

   (d) Mouse anti-MAP2 at a 1:1,000 dilution for neuronal microtubules.

   (e) Mouse anti-Hu at a 1:1,000 dilution for mature neuronal cells.

   (f) Mouse anti-tubulin at a 1:1,000 dilution for microtubules of immature neurons.

   (g) Mouse anti-N-cadherin at a 1:200 dilution for adherens junctions.

   (h) Rabbit anti-Par3 at a 1:200 dilution for cell polarity identification and apical surface identification.

   (i) Rabbit anti-phospho-histone-H3 at a 1:400 dilution for cells in mitosis.

7. Wash cells five times for 5 min in wash buffer.

8. Incubate at RT for 1 h in secondary antibody (use appropriate secondary for primary antigen recognition). Keep protected from light (*see* **Note 7**). All Alexa-fluor secondary antibodies should be diluted 1:1,000 in blocking buffer.

9. Wash cells five times for 5 min in wash buffer.

10. Incubate cells for 10 min, protected from light, in Hoechst 3342 diluted to 1:10,000 in PBS.

11. Wash cells three times for 5 min in PBS.

12. Remove chambers from slide and mount in gelvatol with NPG by applying a drop of gelvatol to the center of the slide and gently placing a glass cover slip on top.

13. Allow mounting medium to set overnight at RT, protected from light.

4   Notes

1. Regionalization and patterning of neural stem cells with proteins, growth factors, and small molecules are typically most effective when provided during the first 10 days of differentiation. This may vary depending on the neuronal subtype of interest.

2. The success of early neural differentiation can be estimated by the morphological appearance of bipolar cells at the periphery of differentiating colonies by D6.

3. Alternatively, poly-L-lysine can be placed on dishes for 2 h at RT so that poly-L-lysine and laminin coating can be done on the same day.

4. Alternatively, Y27632 can be added to the medium and left overnight the day before a planned passage.

5. We prefer using disposable glass shorty pipettes during passages because the length of these pipettes provides greater force for encouraging the cells off the bottom of the dish during passage.

6. Differentiation into mature neuronal cultures takes 60–90 days from D0. Maturation rates can be increased by the addition of small molecules, such as the γ-secretase inhibitor DAPT.

7. We protect cells from light by keeping them contained in large petri dishes covered in foil.

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Direct Conversion of Pluripotent Human Embryonic Stem Cells Under Defined Culture Conditions into Human Neuronal or Cardiomyocyte Cell Therapy Derivatives

Xuejun H. Parsons

Abstract

Developing novel strategies for well-controlled efficiently directing pluripotent human embryonic stem cells (hESCs) exclusively and uniformly towards clinically relevant cell types in a lineage-specific manner is not only crucial for unveiling the molecular and cellular cues that direct human embryogenesis but also vital to harnessing the power of hESC biology for tissue engineering and cell-based therapies. Conventional hESC differentiation methods require uncontrollable simultaneous multi-lineage differentiation of pluripotent cells, which yield embryoid bodies (EB) or aggregates consisting of a mixed population of cell types of three embryonic germ layers, among which only a very small fraction of cells display targeted differentiation, impractical for commercial and clinical applications. Here, a protocol for lineage-specific differentiation of hESCs, maintained under defined culture systems, direct from the pluripotent stage using small-molecule induction exclusively and uniformly to a neural or a cardiac lineage is described. Lineage-specific differentiation of pluripotent hESCs by small-molecule induction enables well-controlled highly efficient direct conversion of nonfunctional pluripotent hESCs into a large supply of high-purity functional human neuronal or cardiomyocyte cell therapy derivatives for commercial and therapeutic uses.

Keywords: Human embryonic stem cells, Lineage-specific differentiation, Defined culture, Neuronal progenitors, Neurons, Cardiac precursors, Cardiomyocytes, Small-molecule induction, Cell therapy, Regenerative medicine

1 Introduction

The successful derivation of human embryonic stem cell (hESC) lines from the in vitro fertilization (IVF) leftover embryos has brought a new era of cellular medicine (1–6). The intrinsic ability of an hESC for both unlimited self-renewal and differentiation into clinically relevant lineages makes it a practically inexhaustible source of replacement cells for human tissue and function restoration. Therefore, it has been regarded as an ideal source to provide a large supply of functional human cells to heal the damaged or the lost tissues that have naturally limited capacity for renewal, such as the human heart and brain. However, pluripotent hESCs themselves are unspecialized nonfunctional cells that cannot be used directly for therapeutic applications in humans. It has been

recognized that pluripotent hESCs must be turned into fate-restricted specialized stem/progenitor/precursor cells or functional mature cells, a process known as differentiation, before use for cell therapy in patients (4–6). How to channel the wide differentiation potential of pluripotent hESCs exclusively and predictably to a desired phenotype has been a major challenge to both developmental study and clinical translation. Conventional hESC differentiation methods require uncontrollable simultaneous multi-lineage differentiation of pluripotent cells, which yield embryoid bodies (EB) or aggregates consisting of a mixed population of cell types of three embryonic germ layers, among which only a very small fraction of cells display targeted differentiation (4–6). Those conventional hESC differentiation methods require laborious, costly, and time-consuming purification or isolation procedures to generate only a small quantity of desired cells, impractical for commercial and clinical applications (4–6). Growing scientific evidences indicate that those conventional methods result in inefficient, instable, and incomplete hESC differentiation and poor performance and high tumor risk of such cell derivatives and tissue engineering constructs following transplantation (4–7). Under conventional protocols presently employed in the field, hESC-derived cellular products consist of a heterogeneous population of mixed cell types, including fully differentiated cells, high levels of various degrees of partially differentiated or uncommitted cells, and low levels of undifferentiated hESCs, posing a constant safety concern when administered to humans (4–6). Without a thorough understanding of the molecular and cellular cues that direct hESC lineage-specific developmental programs, controlled differentiation of pluripotent hESCs effectively and predictably into functional lineages has proven to be one of the daunting challenges for fulfilling the therapeutic promise of hESCs (4–6). In addition, undefined foreign or animal biological supplements and/or feeder cells that have typically been used for the isolation, expansion, and differentiation of hESCs make such cell derivatives unsuitable for clinical applications (4–6). Due to the difficulty of conventional multi-lineage differentiation approaches in obtaining the large number of purified functional human cells, particularly human neurons and cardiomyocytes, typically required for large-scale high-throughput profiling, studies to reveal the mechanism in hESC differentiation towards a functional phenotype remain lacking (5, 8). Developing novel strategies for well-controlled efficiently directing pluripotent hESCs exclusively and uniformly towards clinically relevant cell types in a lineage-specific manner is not only crucial for unveiling the molecular and cellular cues that direct human embryogenesis but also vital to harnessing the power of hESC biology for tissue engineering and cell-based therapies.

Recent breakthrough developments in hESC research have overcome some major obstacles in bringing hESC therapy

derivatives towards human trials, including defined culture systems for de novo derivation and long-term maintenance of clinical-grade stable hESCs and lineage-specific differentiation of pluripotent hESCs by small-molecule induction exclusively and uniformly to a neural or a cardiac lineage (4–6, 9–20). Such novel approach of hESC lineage-specific differentiation direct from the pluripotent stage using small-molecule induction is a major milestone towards clinical application of hESC cell therapy derivatives, enabling highly efficient direct conversion of nonfunctional pluripotent hESCs into a commercial scale of clinical-grade high-purity functional human neuronal or heart muscle cell therapy products as powerful cellular medicines to offer adequate pharmacologic utility and capacity for CNS and heart regeneration in the clinical setting (4–6, 9–20).

Maintaining undifferentiated hESCs in a defined biologics-free culture system that allows faithful expansion and controllable direct differentiation is one of the keys to their therapeutic utility and potential, which requires a better understanding of the minimal essential components necessary for sustaining the pluripotent state and well-being of undifferentiated hESCs (4–6, 9). The hESC lines initially were derived and maintained in co-culture with growth-arrested mouse embryonic fibroblasts (MEFs) (1–4). The need for foreign biologics for derivation, maintenance, and differentiation of hESCs makes direct use of such cells and their derivatives in patients problematic. Without an understanding of the essential developmental components for sustaining hESC pluripotency and self-renewal, such hESC lines are at risk for becoming unhealthy and unstable after prolonged culturing under artificially formulated chemically defined conditions (4–6). To avoid those shortcomings, we have resolved the elements of a defined culture system necessary and sufficient for sustaining the epiblast pluripotency of hESCs, including bFGF, insulin, ascorbic acid, laminin, and activin A, serving as a platform for de novo derivation and long-term maintenance of animal-free therapeutically suitable hESCs and well-controlled efficient specification of such pluripotent cells exclusively and uniformly towards a particular lineage by small-molecule induction (4–6, 9). This defined culture system consists of DMEM/F-12 or knockout-DMEM (KO-DMEM) (80 %), knockout (KO) serum replacement (20 %), L-alanyl-L-gln or L-gln (2 mM), MEM nonessential amino acids (MNAA, 1×), β-mercaptoethanol (β-ME, 100 μM), human purified laminin as the matrix protein, and bFGF (20 ng/ml) (4–6, 9, 15, 17). The knockout serum replacement can be replaced with defined essential factors containing MEM essential amino acids (MEAA, 1×), human insulin (20 μg/ml), ascorbic acid (50 μg/ml), and activin A (50 ng/ml), in which human albumin (10 mg/ml) and human transferrin (8 μg/ml) are added in order to increase cell survival and maintain normal shape and healthy colonies (4–6, 9, 15, 17). Under this defined culture system, >80 % of the hESC colonies remained undifferentiated on surfaces

coated with human laminin, as indicated by their classic undifferentiated morphology; their expression of pluripotent markers, including alkaline phosphatase, Oct-4, SSEA-4, Tra-1-60, and Tra-1-81; their teratoma formation following transplantation; and a normal stable diploid karyotype (4, 6, 9, 15, 17). Genome-wide profiling of chromatin modifications that make up the epigenome of pluripotent hESCs maintained under the defined culture indicated that the broad potential of pluripotent hESCs is defined by an epigenome constituted of open conformation of chromatin mediated by a pattern of Oct-4 global distribution that corresponds genome-wide closely with those of active chromatin modifications, as marked by either acetylated histone H3 or H4 (5, 14, 20). Interestingly, the colonies of undifferentiated cells maintained under the defined culture conditions appeared to be associated with a monolayer of hESC-derived nestin- and vimentin-positive fibroblastic cells that resembled the primitive endoderm (PE) cells surrounding the emerging epiblast in the developing pluripotent inner cell mass (ICM) of the blastocyst in vivo, suggesting that these PE-like cells may spontaneously act as auto feeder layers for the very same undifferentiated hESC colonies from which they were derived, preventing them from differentiating (4, 6, 9, 15, 17). Undifferentiated hESCs maintained under these defined animal-, exogenous feeder-, serum-, and conditioned medium-free conditions could be expanded for prolonged periods in culture, maintain normal karyotype, and form teratomas, suggesting that they remained pluripotent and self-renewing (4–6, 9, 15, 17).

Establishing a defined platform for the long-term stable maintenance of pluripotent hESCs has overcome some of the major obstacles in translational biology. Good manufacturing practice (GMP) quality, defined by both the European Medicine Agency (EMA) and the Food and Drug Administration (FDA), is a requirement for clinical-grade cells, offering optimal defined quality and safety in cell transplantation (6). Resolving minimal essential requirements for the maintenance of pluripotent hESCs allows all poorly characterized and unspecified biological additives, components, and substrates in the culture system, including those derived from animals, to be removed, substituted, or optimized with defined human alternatives for de novo derivation, long-term maintenance, and optimal production of current good manufacturing practice (cGMP)-quality xeno-free hESC lines and their therapy derivatives, which have never been contaminated with animal cells and proteins and, thus, are suitable for therapeutic development and clinical application (4–6, 9–13).

One of the major milestones towards human trials of hESC therapy derivatives is the recent discovery that formulation of minimal essential defined conditions renders pluripotent hESCs be directly and uniformly converted into a specific neural or cardiac lineage by small signal molecule induction (4–6, 9–20). We found

that pluripotent hESCs maintained under the defined culture conditions can be uniformly converted into human neuronal progenitors and neurons by small-molecule induction (4–6, 16–20). Retinoic acid (RA) was identified as sufficient to induce the specification of neuroectoderm direct from the pluripotent state of hESCs maintained under the defined culture, without going through a multi-lineage EB stage, and trigger a cascade of neuronal lineage-specific progression to human neuronal progenitors (Xcel-hNuP or hESC-I hNuP) and neurons (Xcel-hNu or hESC-I hNu) of the developing CNS in high efficiency, purity, and neuronal lineage specificity by promoting nuclear translocation of the neuronal specific transcription factor Nurr-1 (4–6, 16–20) (Figs. 1 and 2). Upon exposure of undifferentiated hESCs maintained in the defined culture to RA, all the cells within the colony underwent morphology changes to large differentiated cells that ceased
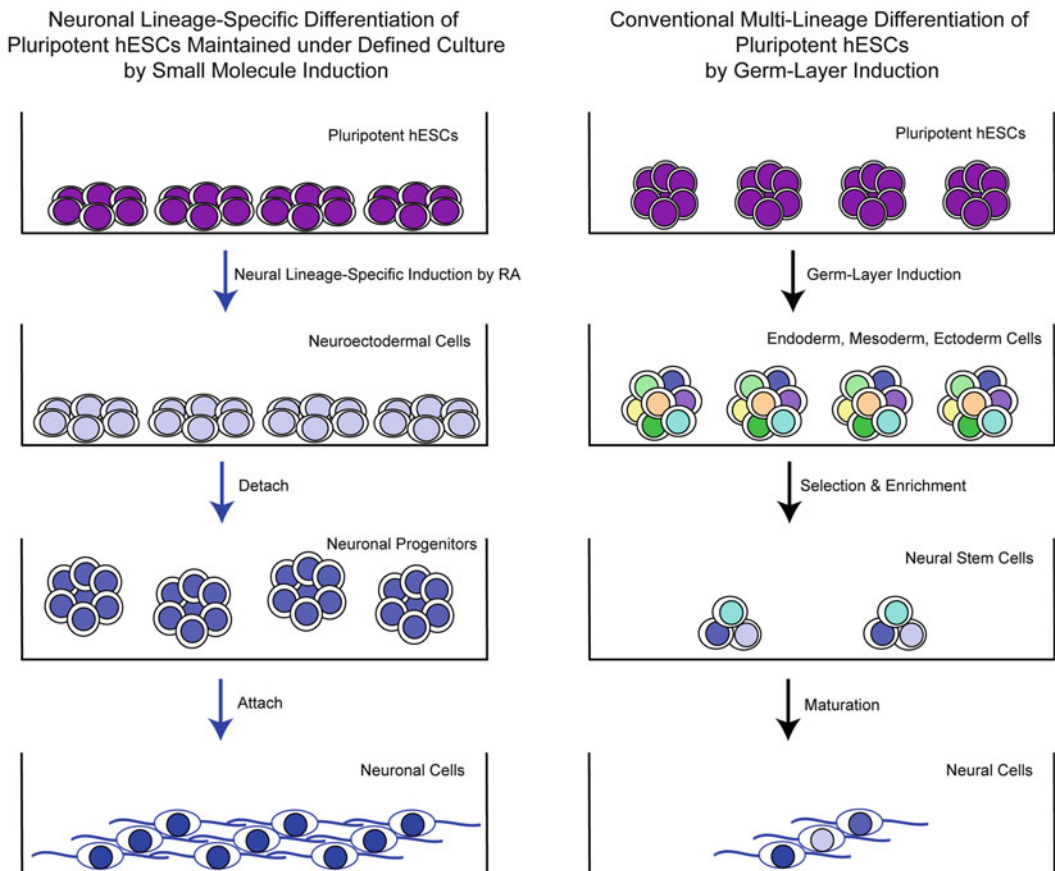
Fig. 1 Schematic comparison of well-controlled efficient neuronal lineage-specific differentiation of pluripotent hESCs maintained under defined culture exclusively to a neuronal fate by small signal molecule induction versus conventional neural differentiation approach using multi-lineage inclination of pluripotent cells through spontaneous germ layer induction (by courtesy of Parsons, Br. Biotechnol. J. 2013;3(4):424–457 (6))
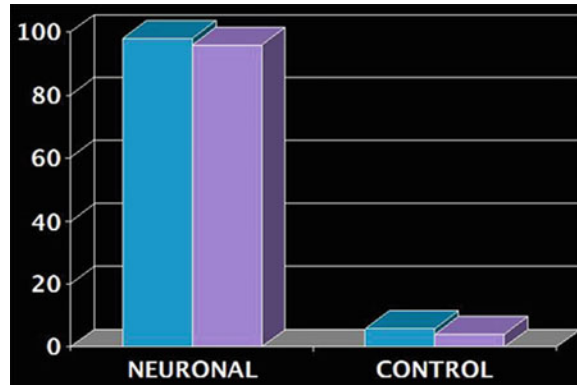
Fig. 2 Comparison of neuronal differentiation efficiencies of pluripotent hESCs by small molecule-induced neuronal lineage-specific differentiation (NEURONAL) with conventional multi-lineage differentiation through germ layer induction (CONTROL)

expressing pluripotency-associated markers (e.g., Oct-4) and began expressing neuroectoderm-associated markers, but not markers associated with other lineages (stage 1—human neuroectodermal cells) (4, 6, 17) (Fig. 1). These differentiated cells continued to multiply, and the colonies increased in size, proceeding spontaneously to express the early neuronal marker β-III-tubulin (4, 6, 17). The more mature neuronal marker Map-2 began to appear in areas of the colonies where cells had piled up (4, 6, 17). After detached, these differentiating hESCs then formed neuroblasts that were uniformly positive for β-III-tubulin in suspension (stage 2—human neuronal progenitor cells [hESC-I hNuP]) (4, 6, 17) (Fig. 1). Upon removal of bFGF and after permitting the neuroblasts to attach in neural differentiation media containing VEGF (20 ng/ml), NT-3 (10 ng/ml), and BDNF (10 ng/ml), β-III-tubulin- and Map-2-expressing, exuberantly neurite-bearing cells and pigmented cells (typical of those in the ventral mesencephalon) began to appear with a drastic increase in efficiency (>90 %) (stage 3—human neuronal cells in the developing CNS [hESC-I hNu]) when compared to similarly cultured cells derived from untreated EBs (<5 %) (4, 6, 17) (Figs. 1, 2, and 3). These in vitro neuroectoderm-derived human neuronal progenitors (hESC-I hNuP) yielded neurons efficiently and exclusively, suggesting that neuroectoderm specification transforms pluripotent hESCs uniformly into a more neuronal lineage-specific nuclear Nurr1-positive embryonic neuronal progenitor than the prototypical neuroepithelial-like nestin-positive human neural stem cells (hNSCs) derived either from CNS or hESCs (4–6, 16–20). Genome-scale profiling of microRNA (miRNA) differential expression showed that the expression of pluripotency-associated hsa-miR-302 family was silenced and the
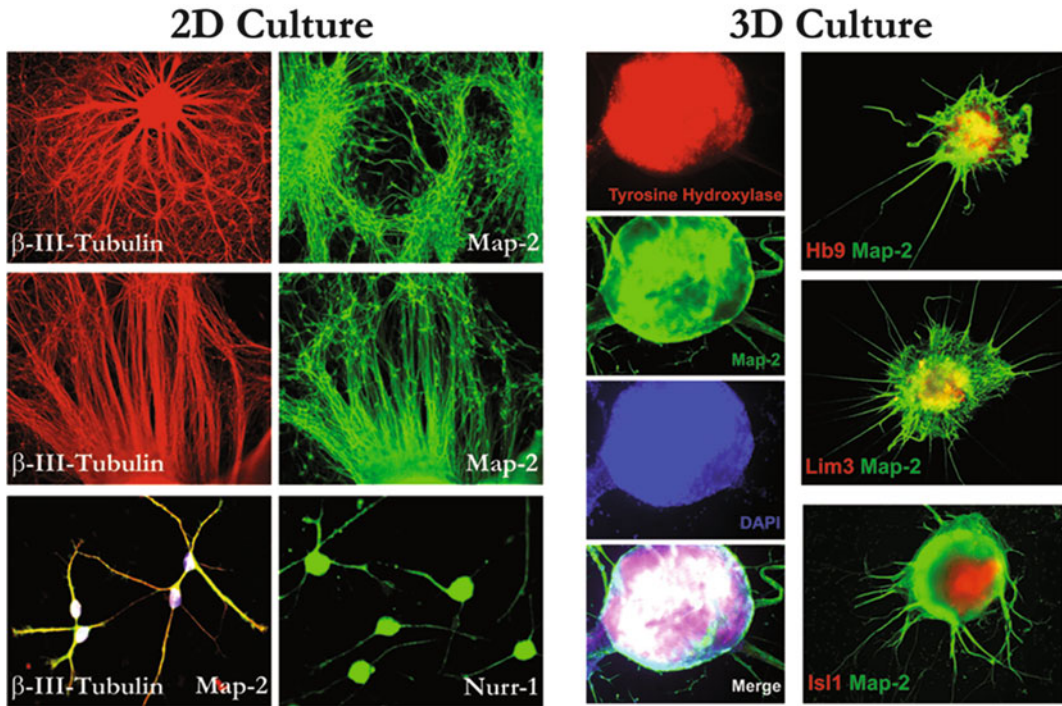
Fig. 3 Images of human neuronal cells (Xcel-hNu or hESC-I hNu) derived from hESCs by small molecule-induced neuronal lineage-specific differentiation

expression of Hox miRNA hsa-miR-10 family that regulates gene expression predominantly in neuroectoderm was induced to high levels in these hESC-derived neuronal progenitors (16, 19) (Fig. 4). Following transplantation, these hESC neuronal derivatives engrafted widely and yielded well-dispersed and well-integrated human neurons at a high prevalence within neurogenic regions of the brain (18, 19). Under neuronal subtype specification conditions in 3D culture, these hESC-derived neuronal cells by small-molecule induction further proceeded to express subtype neuronal markers associated with ventrally located neuronal populations, such as dopaminergic (DA) neurons and motor neurons, demonstrating their therapeutic potential for regeneration of CNS neuronal cell types and subtypes in vivo as stem cell therapy to be translated to patients in clinical trials (4, 6, 18, 19) (Fig. 3). This technology breakthrough enables neuronal lineage-specific differentiation direct from the pluripotent state of hESCs with small-molecule induction, providing a much-needed in vitro model system for investigating molecular controls in human CNS development in embryogenesis as well as a large supply of clinical-grade human neuronal cells across the spectrum of developmental stages for tissue engineering and cell therapy against CNS disorders (4–6).
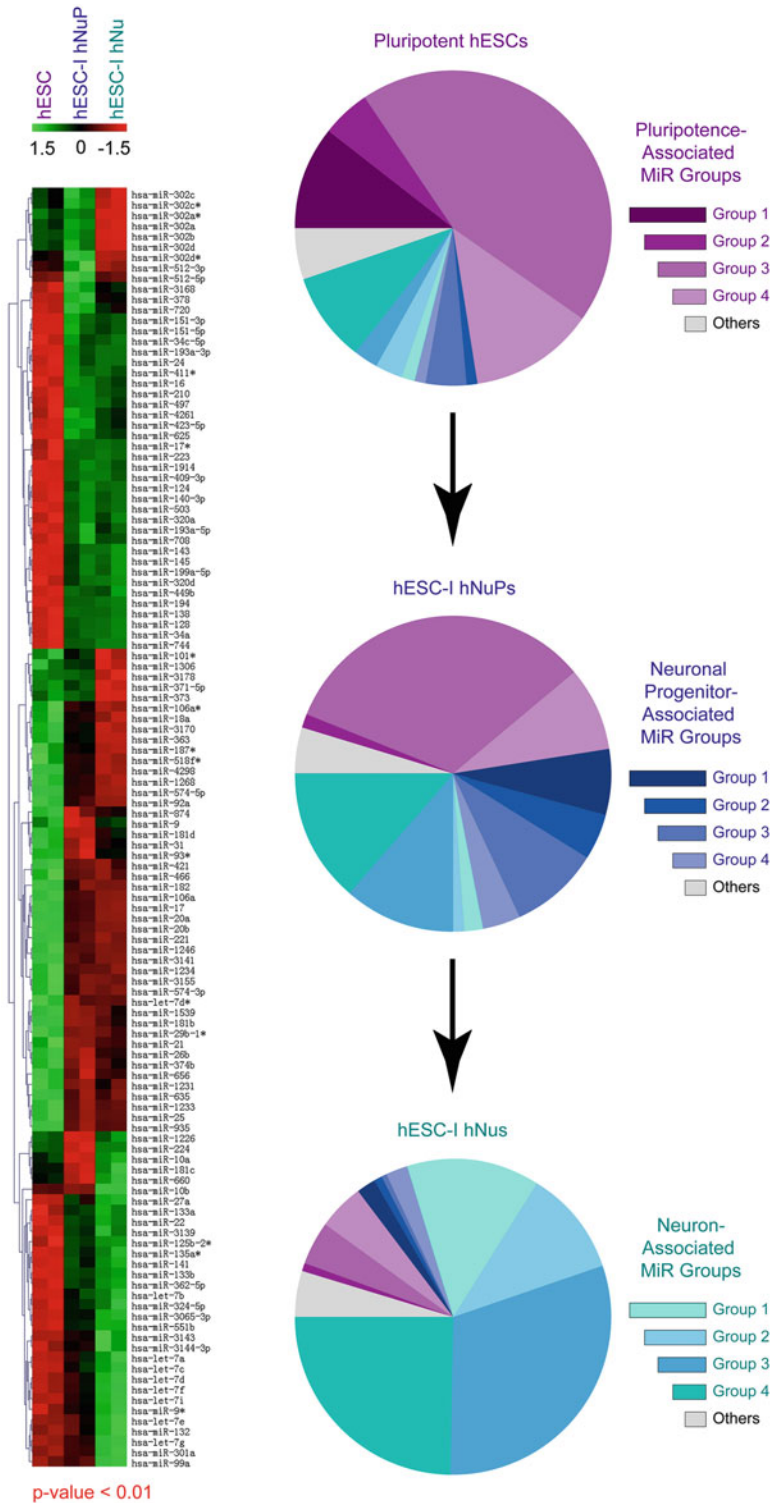
Fig. 4 MiRNA (MiR) signatures of human neuronal progenitor cells (Xcel-hNuP or hESC-I hNuP) and human neuronal cells (Xcel-hNu or hESC-I hNu) derived from hESCs by small molecule-induced neuronal lineage-specific differentiation (by courtesy of Parsons et al., Mol. Med. Ther. 2013;1:2 (19))
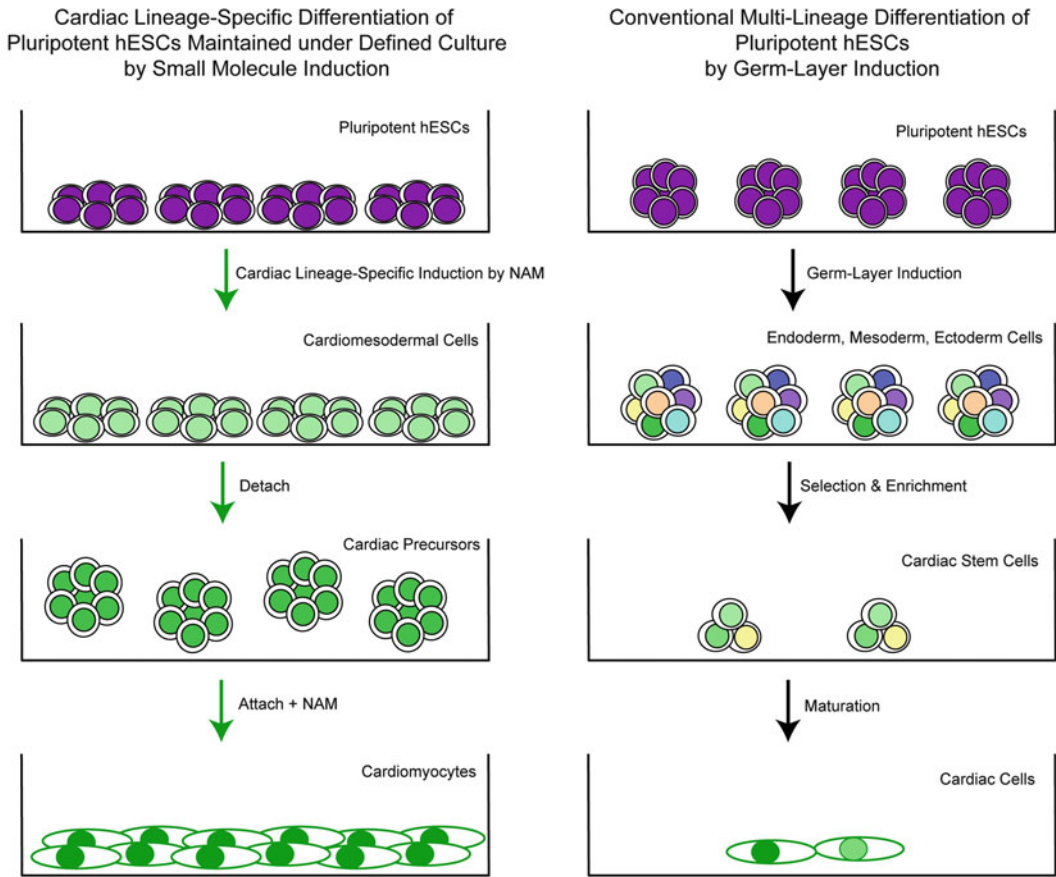
Fig. 5 Schematic comparison of well-controlled efficient cardiac lineage-specific differentiation of pluripotent hESCs maintained under the defined culture exclusively to a cardiac fate by small signal molecule induction versus conventional cardiac differentiation approach using multi-lineage inclination of pluripotent cells through spontaneous germ layer induction (by courtesy of Parsons, Br. Biotechnol. J. 2013;3(4):424–457 (6))

Similarly, we found that formulation of minimal essential defined conditions for hESCs rendered small molecule nicotinamide (NAM) sufficient to induce the specification of cardiomesoderm direct from the pluripotent state of hESCs by promoting the expression of the earliest cardiac specific transcription factor Csx/Nkx2.5 and triggering progression to cardiac precursors (Xcel-hCardP or hESC-I hCardP) and beating cardiomyocytes (Xcel-hCM or hESC-I hCM) with high efficiency (4–6, 9–16) (Figs. 5 and 6). Upon exposure of undifferentiated hESCs maintained in the defined culture to NAM, all the cells within the colony underwent morphology changes to large differentiated cells that downregulated the expression of pluripotency-associated markers (e.g., Oct-4, Sox-2) and began expressing the earliest marker for heart precursor (e.g., Nkx2.5, α-actinin), but not markers associated with other lineages (stage 1—human cardiomesodermal cells)
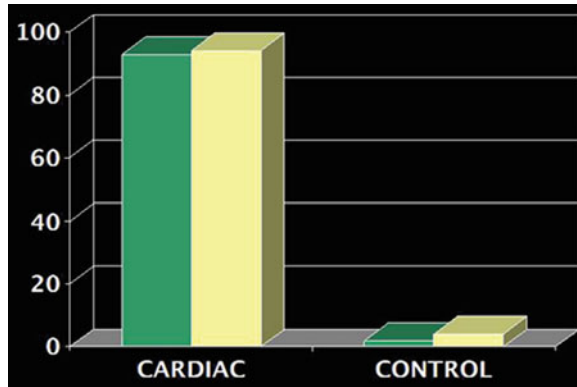
Fig. 6 Comparison of cardiac differentiation efficiencies of pluripotent hESCs by small molecule-induced cardiac lineage-specific differentiation (CARDIAC) with conventional multi-lineage differentiation through germ layer induction (CONTROL)

(4, 6, 9, 15) (Fig. 5). These large differentiated cells continued to multiply, and the colonies increased in size (4, 6, 9, 15). Increased intensity of Nkx2.5 was usually observed in areas of the colonies where cells began to pile up (4, 6, 9, 15). After detached, these differentiating hESCs then formed cardioblasts that uniformly expressed Nkx2.5 in suspension (stage 2—human cardiac precursor cells) (4, 6, 9, 15) (Fig. 5). After permitting the cardioblasts to attach and further treating them with NAM, beating cardiomyocytes began to appear after withdrawal of NAM with a drastic increase in efficiency when compared to similarly cultured cells derived from untreated EBs (stage 3—human cardiomyocytes) (4, 6, 9, 15) (Fig. 5). Cells within the beating cardiospheres expressed markers characteristic of cardiomyocytes, including Nkx2.5, GATA-4, α-actinin, cardiac troponin I (cTnI), and cardiac troponin T (cTnT) (4, 6, 9, 15). Electrical profiles of the cardiomyocytes confirmed their contractions to be strong rhythmic impulses reminiscent of the p-QRS-T-complexes seen from body surface electrodes in clinical electrocardiograms (6, 15) (Fig. 7). NAM induced global histone deacetylation and nuclear translocation of the class III NAD-dependent histone deacetylase SIRT1, suggesting that NAM triggers the activation of SIRT1 and NAD-dependent histone de-acetylation that lead to global chromatin silencing yet selective activation of a subset of cardiac specific genes and subsequently cardiac fate determination of pluripotent hESCs (5, 16, 21). This technology breakthrough enables cardiac lineage-specific differentiation direct from the pluripotent state of hESCs with small-molecule induction, providing a much-needed in vitro hESC model system for investigating molecular controls in human embryonic heart formation as well as a large supply of human cardiomyocyte precursors and cardiomyocytes for myocardium regeneration (4–6, 9–13).
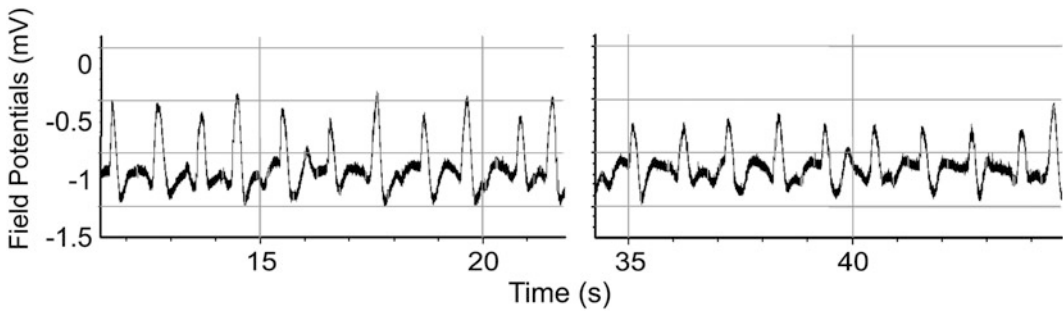
Fig. 7 Electrical profiles of the cardiomyocytes derived from hESCs by small molecule-induced cardiac lineage-specific differentiation shows strong rhythmic contractions reminiscent of the p-QRS-T-complexes seen from body surface electrodes in clinical electrocardiograms (by courtesy of Parsons et al., J. Vis. Exp. 2011;57:e3274 (15))

Derivation of hESCs provides a powerful in vitro model system to investigate the molecular controls in human embryonic development as well as an unlimited source to generate the diversity of human cell types and subtypes across the spectrum of development stages for repair (4–6). Lineage-specific differentiation of pluripotent hESCs by small-molecule induction enables highly efficient direct conversion of hESCs from the pluripotent stage into a commercial scale of neural or cardiac lineage-specific cell therapy derivatives, which is a major milestone towards human trials of hESC cell therapy derivatives, offering the benefits in efficiency, stability, safety, efficacy, and large-scale production of clinical-grade high-quality human cell therapy products in cGMP facility for commercial and therapeutic uses over all other existing approaches. Such breakthrough stem cell technologies have demonstrated the direct pharmacologic utility and capacity of hESC cell therapy derivatives for human CNS and myocardium regeneration and, thus, presented the hESC cell therapy derivatives as a powerful pharmacologic agent of cellular entity for CNS and heart repair. Currently, such hESC neuronal and cardiomyocyte cell therapy derivatives or products are the only available human cell sources in commercial scales with adequate capacity to regenerate the CNS neuron and contractile heart muscle, vital for CNS and heart repair for a wide range of neurological and cardiovascular diseases in the clinical setting. Lineage-specific differentiation of pluripotent hESCs by small-molecule induction opens the door to investigate molecular embryogenesis in human development. Unveiling genetic and epigenetic programs embedded in hESC lineage specification will not only contribute tremendously to our knowledge regarding molecular embryogenesis in human development but also allow direct control and modulation of the pluripotent fate of hESCs when deriving an unlimited supply of clinically relevant lineages for regenerative medicine (5, 6). Embedding lineage-specific genetic and epigenetic developmental programs into the open epigenomic

landscape of pluripotent hESCs will offer a new repository of human stem cell therapy derivatives for the future of regenerative medicine (5, 6). Transforming nonfunctional pluripotent hESCs into fate-restricted functional human cell therapy derivatives or products dramatically increases the clinical efficacy of graft-dependent repair and safety of hESC-derived cellular products, marking a turning point in cell-based regenerative medicine from current studies in animals towards human trials (4–6, 10–13).

Here, a protocol for lineage-specific differentiation of hESCs, maintained under defined culture systems, direct from the pluripotent stage using small-molecule induction exclusively and uniformly to a neural or a cardiac lineage is described (4–6, 9–20). Lineage-specific differentiation of pluripotent hESCs by small-molecule induction enables well-controlled highly efficient direct conversion of nonfunctional pluripotent hESCs into a large supply of high-purity functional human neuronal or cardiomyocyte cell therapy derivatives for commercial and therapeutic uses (4–6, 9–20).

2   Materials

2.1   Solution and Media Preparation

1. Gelatin coating solution: 0.1 % (w/v) gelatin in ddH$_2$O, autoclaved and stored at 4 °C.

2. Matrigel coating stock solutions: Slow thaw (*see* **Note 1**) Matrigel (growth factor reduced, 10 ml) at 4 °C overnight, add 10 ml ice-cold DMEM or DMEM/F12, mix well, aliquot 1 ml/tube underneath sterilized tissue culture hood, and store at −20 °C.

3. Matrigel coating working solution: Slow thaw 1 ml Matrigel coating stock solution aliquot at 4 °C for 1–2 h, transfer to 14 ml chilled DMEM or DMEM/F12, and mix well underneath sterilized tissue culture hood immediately before coating.

4. Human laminin coating solution: Dilute 1 ml human laminin solution (0.5 mg/ml in Tris-buffered NaCl, store at −80 °C, slow thaw at 4 °C for 1–2 h) (*see* **Note 1**) with ice-cold DMEM or DMEM/F12 to 12.5 ml working solution (40 μg/ml) underneath sterilized tissue culture hood immediately before coating.

5. Growth factor stock solutions (500–1,000×): Dissolve growth factor at 10 μg/ml in sterilized buffer (0.5 % BSA, 1.0 mM DTT, 10 % glycerol, 1× PBS) and store as 50–100 μl/tube aliquots at −80 °C.

6. All-trans-retinoic acid working solution (1,000×): 10 mM in DMSO, store in small aliquots at −80 °C.

7. HESC media: DMEM/F12 or KO-DMEM (80 %), KO serum replacement (20 %), L-alanyl-L-gln or L-gln (2 mM), MNAA (1×), and β-ME (100 μM), filtered and stored at 4 °C, supplemented with 20 ng/ml bFGF immediately before use. Or replacing "KO serum replacement" with defined components: DMEM/F12 or KO-DMEM (100 %), L-alanyl-L-gln or L-gln (2 mM), MNAA (1×), MEAA (1×), and β-ME (100 μM), filtered and stored at 4 °C, supplemented with bFGF (20 ng/ml), human insulin (20 μg/ml), ascorbic acid (50 μg/ml), human activin A (50 ng/ml), human albumin/Albumax (10 mg/ml), and human transferrin (8 μg/ml) immediately before use (*see* **Note 2**).

8. HNSC media: DMEM/F12 (100 %), N-2 supplement (1×), heparin (8 μg/ml).

9. HESC cardiac differentiation media: DMEM/F12 (90 %), defined FBS (10 %) (*see* **Note 3**), and L-alanyl-L-gln or L-gln (2 mM).

10. Media containing NAM: Add NAM to hESC media or hESC cardiac differentiation media at a final concentration of 10 mM, filter, store at 4 °C, and use within 2 weeks of preparation.

2.2 Plate Coating

1. Coat plates with gelatin: Add 2.5 ml/well of gelatin solution to 6-well plates and incubate overnight in a 37 °C humidified incubator.

2. Coat plates with laminin: Chill gelatin-coated plates, remove gelatin, add 2.5 ml/well Matrigel or human laminin coating working solution to pre-chilled gelatinized 6-well plates, and incubate at 4 °C overnight.

3 Methods

All procedures are performed under a sterilized tissue culture hood or sterilized conditions (*see* **Note 4**).

3.1 Passaging and Seeding Undifferentiated hESCs Under Defined Culture Conditions

1. Allow undifferentiated hESC colonies (*see* **Note 5**) to grow to 5–7 days old, and take hESC culture plate to dissecting microscope (pre-warm dissecting stage to 37 °C) underneath dissecting sterilized hood.

2. Select colonies with more than 75 % undifferentiated hESCs (*see* **Note 6**) to be split. These hESC colonies are usually slightly opaque with defined edges (*see* **Note 7**). Colonies containing piled-up cells, an indication of the beginning of differentiation, usually appear to be white. Colonies containing mostly differentiated cells usually appear to be clear. Do not select white or clear colonies.

3. Carefully outline the selected colonies with the edge of P2 sterile pipette tip or pulled glass capillary underneath a dissecting microscope with a warmed dissecting stage (*see* **Note 8**).

4. Along the outlines, use the edge of a sterile P2 pipette tip or pulled glass capillary to detach the surrounding fibroblast layer from the hESC colony. Then remove the differentiated fibroblast cell layer surrounding the colony, and also remove all the differentiated parts of the colony with the edge of P2 sterile pipette tip or pulled glass capillary.

5. Remove the old media containing floating detached differentiated cells by aspirating. Wash with hESC media (without bFGF) once. Add 3 ml/well fresh hESC media containing 20 ng/ml bFGF.

6. Cut the undifferentiated hESC colonies into small pieces, and detach with sterile pipette tip or pulled glass capillary.

7. Pool the media containing detached colony pieces together in a 50 ml conical tube. Wash the plate once with 1 ml/well hESC media containing 20 ng/ml bFGF and pool together.

8. Aspirate the Matrigel or human laminin solution from the coated fresh plates. Aliquot 4 ml/well hESC media containing colony pieces to a 6-well plate. Gently transfer the plate to incubator without shaking, and allow colony pieces to seed overnight without disturbing in a humidified 37 °C incubator with an atmosphere of 5 % CO_2.

9. Replace hESC media every other day (*see* **Note 9**). hESCs can be split at a ratio of 1:4 to 1:8 every week.

3.2 Direct Conversion of Pluripotent hESCs into Neuronal Fate-Restricted Cell Therapy Derivatives

1. At day 3 after seeding of undifferentiated hESCs, remove most of the old media from each well of the plate and leave enough media to allow hESC colonies to be submerged (never allow hESCs to dry out). Replace with 4 ml/well fresh hESC media containing 20 ng/ml bFGF and 10 μM RA (*see* **Note 10**).

2. Replace old media with fresh hESC media containing 20 ng/ml bFGF and 10 μM RA every other day, and allow neural induced hESC colonies to grow to day 7 or 8. All the cells within the colony will undergo morphology changes to large differentiated cells that will continue to multiply (*see* **Note 11**). The colonies will increase in size to cover the plate by day 7 or 8, and cells will begin to pile up in some areas of the colonies (*see* **Note 12**).

3. Take hESC culture plate to dissecting microscope (pre-warm dissecting stage to 37 °C) underneath dissecting sterilized hood. Carefully outline the colonies, and remove fibroblast layer surrounding the colony (differentiated cells migrated

out of the colony) with the edge of P2 sterile pipette tip or pulled glass capillary (*see* **Note 8**).

4. Remove the old media containing floating detached fibroblast cells by aspirating. Wash with hESC media (without bFGF) once. Add 3 ml/well fresh hESC media (without bFGF).

5. Cut the neural induced hESC colonies into small pieces and detach with sterile pipette tip or pulled glass capillary.

6. Pool the media containing detached colony pieces together in a 50 ml conical tube. Wash the plate once with 1 ml/well hESC media (without bFGF) and pool together.

7. Aliquot 4 ml/well serum-free hESC media containing colony pieces to a 6-well ultralow attachment plate and incubate in a 37 °C humidified incubator to allow floating cellular clusters (neuroblasts) to form in a suspension culture for 4–5 days.

8. Pool the media containing floating neuroblasts together in a 50 ml conical tube. Centrifuge at 1,400 rpm for 5 min. Aspirate the old media as much as you can, and add equal amount of fresh hNSC media containing freshly added VEGF (20 ng/ml), NT-3 (10 ng/ml), and BDNF (10 ng/ml).

9. Pipette up and down to mix floating neuroblasts, and aliquot 4 ml/well to 6-well plates. The neuroblasts can also be seeded in a laminin/collagen (Matrigel)- or human laminin-polymerized three-dimensional matrix in serum-free hNSC media containing VEGF (20 ng/ml), NT-3 (10 ng/ml), and BDNF (10 ng/ml). Transfer the plates to a 37 °C humidified incubator to allow neuroblasts to attach overnight.

10. Replace hNSC media containing VEGF (20 ng/ml), NT-3 (10 ng/ml), and BDNF (10 ng/ml) every other day. Extensive networks of neurite-bearing neuronal cells and pigmented cells, typical of the CNS, will begin to appear within 2 weeks of continuous cultivation and increase in numbers with time and could be sustained for over 3 months (*see* **Note 13**).

3.3 Direct Conversion of Pluripotent hESCs into Cardiac Fate-Restricted Cell Therapy Derivatives

1. At day 3 after seeding of undifferentiated hESCs, remove most of the old media from each well of the plate and leave enough media to allow hESC colonies to be submerged (never allow hESCs to dry out). Replace with 4 ml/well fresh hESC media containing 20 ng/ml bFGF and 10 mM NAM (*see* **Note 14**).

2. Replace old media with fresh hESC media containing 20 ng/ml bFGF and 10 mM NAM every other day, and allow cardiac induced hESC colonies to grow to days 8–10. Upon exposing to NAM, all the cells within the colony will undergo morphology changes to large differentiated cells that will continue to multiply (*see* **Note 15**). The colonies will increase in size to cover the plate by days 8–10, and cells will begin to pile up in some areas of the colonies (*see* **Note 16**).

3. Take hESC culture plate to dissecting microscope (pre-warm dissecting stage to 37 °C) underneath dissecting sterilized hood. Carefully outline the colonies, and remove fibroblast layer surrounding the colony (differentiated cells migrated out of the colony) with the edge of P2 sterile pipette tip or pulled glass capillary (*see* **Note 8**).

4. Remove the old media containing floating detached fibroblast cells by aspirating. Wash with hESC media (without bFGF) once. Add 3 ml/well fresh hESC media (without bFGF).

5. Cut the cardiac induced hESC colonies into small pieces and detach with sterile pipette tip or pulled glass capillary.

6. Pool the media containing detached colony pieces together in a 50 ml conical tube. Wash the plate once with 1 ml/well hESC media (without bFGF) and pool together.

7. Aliquot 4 ml/well serum-free hESC media containing colony pieces to a 6-well ultralow attachment plate and incubate in a 37 °C humidified incubator to allow floating cellular clusters (cardioblasts) to form for 4–5 days.

8. Pool the media containing floating cardioblasts together in a 50 ml conical tube. Centrifuge at 1,400 rpm for 5 min. Aspirate the old media as much as you can, and add equal amount of fresh hESC cardiac differentiation media containing 10 mM NAM.

9. Pipette up and down to mix floating cardioblasts and aliquot 4 ml/well to 6-well plates. Transfer the plates to a 37 °C humidified incubator to allow cardioblasts to attach overnight.

10. Replace hESC cardiac differentiation media containing 10 mM NAM every other day.

11. At day 8, replace with hESC cardiac differentiation media without NAM, and continue to replace hESC cardiac differentiation media every other day. Beating cardiomyocytes will begin to appear in about 2 weeks of continuous cultivation after withdrawal of NAM and increase in numbers with time and could retain strong and rhythmic contractions for over 3 months (*see* **Note 17**).

4 Notes

1. There are a few steps to be careful with when preparing stock solutions. Take care to slow thaw Matrigel and human laminin (stored at −80 °C) at 4 °C before its use.

2. When preparing hESC media, some of the supplements are added just before it is used and not any sooner. These include bFGF, ascorbic acid, human insulin, activin A, and transferrin.

3. Defined FBS from Hyclone (SH30073-03): The quality of defined FBS may vary depending on the sources and batches, which may affect hESC cardiac differentiation.

4. Generally, individuals new to this method will struggle, because it requires training and handling experience of culture techniques for hESCs. Training and visual demonstration of this method are critical, because many factors and the slightest changes of culture conditions could potentially influence the quality of hESCs and induce unwanted stress on the cells that may cause the loss of pluripotency and differentiation of the cells into unwanted lineages (see ref. 15 and 17 for visual demonstration).

5. Ensuing cell lines free of pathogens and mycoplasma should always be taken before performing this procedure.

6. The undifferentiated hESCs will form the typical tightly packed colonies of small compact cells that are Oct-4 positive with defined edges, while large differentiated cells will migrate outside the colonies and become Oct-4 negative. The classic undifferentiated morphology of hESC colonies is consistent with their expression of pluripotent markers, including alkaline phosphatase, Oct-4, SSEA-4, Tra-1-60, and Tra-1-81, by immunocytochemical analysis.

7. Pluripotent hESCs are at a transient stage and undergo spontaneous differentiation even under optimal conditions. Although preferred, it is difficult to ascertain that these cells are 100 % undifferentiated under dissecting microscope. >75 % undifferentiated is usually considered as the pass point. The differentiated cells usually will not seed and continue to grow and, thus, will be eliminated in the passaging process.

8. If a dissecting microscope with a warmed dissecting stage is not available, alternately, the plates can be held up to the light and the slightly opaque colonies can be outlined on the bottom of the plates using a marker.

9. Approximately every 2 days, remove most of the old media from each well or dish, and leave enough media to allow hESC colonies to be submerged (never allow hESCs to dry out). Replace with 4 ml/well fresh hESC media containing 20 ng/ml freshly added bFGF.

10. The main advantage of this technique over existing methods of multi-lineage differentiation of pluripotent cells is that, in other techniques, only a small fraction of cells pursue a neuronal phenotype. However, our method allows well-controlled efficient induction of pluripotent hESCs exclusively and uniformly towards a neuronal lineage by simple provision of small molecules.

11. RA-treated large differentiated cells inside the colonies are Oct-4 negative and begin to express various neuroectoderm-associated markers, including HNK1, AP2, and TrkC.

Coincident with the appearance of the neuroectodermal cells, the neuronal specific transcriptional factor Nurr-1, implicated in dopaminergic neuronal differentiation and activation of the tyrosine hydroxylase gene, translocates to the nucleus.

12. These large differentiated cells will continue to multiply, and the colonies will increase in size, spontaneously expressing the early neuronal marker beta-III-tubulin. The more mature neuronal marker Map-2 will begin to appear in areas of the colonies where cells have piled up.

13. These hESC cell-derived neuronal cells will express beta-III-tubulin and co-express Map-2. Once mastered, this protocol can be used to generate human neuronal progenitors and human neuronal cells uniformly from pluripotent hESCs in about 4 weeks if it is performed properly.

14. The main advantage of this technique is that it allows efficient induction of pluripotent hESCs exclusively towards a cardiac lineage by simple provision of small molecules. However, in existing methods of multi-lineage differentiation, only a small fraction of cells pursue a cardiac phenotype.

15. Upon exposure of undifferentiated hESCs to NAM, all the cells within the colony will undergo morphological changes into large differentiated cells that show weak expression of Oct-4 or are Oct-4 negative. These cells will begin to express the cardiac specific transcription factor (Csx) Nkx2.5 and also express alpha-actinin. Both characteristics are consistent with a cardiomesoderm phenotype.

16. These differentiated cells will continue to multiply, and the colonies will increase in size. In areas of the colonies where cells have piled up, Nkx2.5 expression will become more intense.

17. Beating cardiomyocytes will appear mostly as large cellular clusters with rhythmic contractions. Cells within the beating cardiomyocyte clusters will uniformly express markers characteristic of cardiomyocytes, including Nkx2.5 and alpha-actinin. Once mastered, this technique can be used to generate human cardiac precursors and human beating cardiomyocytes uniformly from pluripotent hESCs in about 5 weeks if it is performed properly.

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Generation of Epithelial Cell Populations from Human Pluripotent Stem Cells Using a Small-Molecule Inhibitor of Src Family Kinases

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Abstract

Human pluripotent stem cells (hPSCs), under the right conditions, can be engineered to generate populations of any somatic cell type. Knowledge of what mechanisms govern differentiation towards a particular lineage is often quite useful for efficiently producing somatic cell populations from hPSCs. Here, we have outlined a strategy for deriving populations of simple epithelial cells, as well as more mature epidermal keratinocyte progenitors, from hPSCs by exploiting a mechanism previously shown to direct epithelial differentiation of hPSCs. Specifically, we describe how to direct epithelial differentiation of hPSCs using an Src family kinase inhibitor, SU6656, which has been shown to modulate β-catenin translocation to the cell membrane and thus promote epithelial differentiation. The differentiation platform outlined here produces cells with the ability to terminally differentiate to epidermal keratinocytes in culture through a stable simple epithelial cell intermediate that can be expanded in culture for numerous (>10) passages.

Keywords: Human pluripotent stem cells, Differentiation, Epithelial cells, Src kinase inhibitor, Epidermal

1 Introduction

Human pluripotent stem cells (hPSCs) can be coerced into generating populations of any somatic cell type in the human body (1–3). Given this ability, hPSCs can be used for in vitro models to study human development and disease as well as for applications in regenerative medicine. To generate populations of somatic cells to be used for such applications, it is imperative to design differentiation systems that are robust and produce high-purity populations of cells. While there are different strategies to obtain epithelial populations from hPSCs (4–8), a recent study demonstrated how epithelial differentiation can be modulated by β-catenin localization, providing insight as to what mechanisms are involved in governing the epithelial differentiation process (9). Here, we describe a method to produce simple epithelial cells and, subsequently, epidermal keratinocyte progenitor populations by exploiting this mechanism using an Src family kinase inhibitor.

To efficiently derive populations of epithelial cells to be used for tissue engineering applications, it is optimal first to generate highly enriched populations of simple epithelial cells. These cells can be characterized by high levels of cytokeratin 18 (K18), expressed by simple, or single-layered epithelial cells in vivo (8), and the lack of transcription factors such as Oct4 and Nanog, expressed in hPSCs and which play critical roles in regulating pluripotency (10). Upon further differentiation and epithelial maturation, simple epithelial cells lose K18 expression and acquire expression of cytokeratin 14 (K14), found in the basal layer of many epithelial tissues, including the epidermis (8, 11). In addition, the transcription factor, p63, which plays a role in the regenerative ability of many epithelial tissues, is expressed during and throughout epithelial differentiation (12–14). Cells can be monitored using assays such as immunofluorescence and flow cytometry to detect these marker proteins representing cells at various stages of differentiation and to ensure that populations of cells generated from hPSCs are highly enriched in epithelial cells for future incorporation into tissue constructs for various clinical and research applications.

2   Materials

2.1   Cell Growth and Differentiation

1. hPSC growth medium: mTeSR1 (STEMCELL Technologies, Vancouver, Canada).

2. hPSC differentiation medium 1: Dulbecco's Modified Eagle's Medium (DMEM)/F12 (1:1) supplemented with 20 % knock-out serum replacer (KSR), 1× nonessential amino acids (NEAA), 1 mM L-glutamine (all from Life Technologies, Carlsbad, CA), 0.1 mM β-mercaptoethanol (Sigma, St. Louis, MO), and 6 μM SU6656 (Sigma).

3. hPSC differentiation medium 2: DMEM/F12 (1:1) supplemented with 20 % KSR, 1× NEAA, 1 mM L-glutamine (all from Life Technologies), 0.1 mM β-mercaptoethanol (Sigma), 1 μM retinoic acid (RA, Sigma), and 10 ng/ml bone morphogenetic protein 4 (BMP4, Life Technologies).

4. Matrigel (BD, Biosciences, San Jose, CA). Store at −80 °C in single-use aliquots. Thaw at 4 °C. All manipulations must be conducted on ice using chilled pipette tips to avoid gelation of Matrigel solution. To coat a 6-well plate with Matrigel, dissolve 0.5 mg of Matrigel (solution) in 6 ml of DMEM/F12 and coat each well with 1 ml of solution. Allow Matrigel to gel at 37 °C for at least 1 h prior to plating cells.

5. Dispase (Life Technologies): Reconstituted in DMEM/F12 at 2 mg/ml. Store aliquots at −20 °C.

6. Gelatin powder (Sigma) dissolved in water at 0.1 % (w/v): To coat a 6-well plate with gelatin, coat each well with 1 ml of gelatin solution and store at 37 °C for at least 4 h prior to plating cells.

7. Defined keratinocyte serum-free medium (K-DSFM) and supplement (Life Technologies).

8. Epithelial cell expansion medium: K-DSFM supplemented with 5 % fetal bovine serum (both from Life Technologies).

9. ROCK inhibitor Y27632 (Sigma): Add to culture medium for a final concentration of 10 μM.

10. Trypsin (0.05 %)–ethylenediamine tetraacetic acid (EDTA, 1 mM, Life Technologies).

11. Accutase (Life Technologies).

12. Versene (Life Technologies).

2.2 Immuno-fluorescent Staining

1. IF fixation buffer: 16 % (w/v) paraformaldehyde (PFA, Sigma) diluted to 4 % (v/v) in PBS.

2. Blocking buffer: PBS with 5 % milk or chick serum (Sigma) and 0.4 % (v/v) Triton X-100 (Fisher, Pittsburgh, PA) added.

3. Primary antibodies (recommended dilution): Rabbit anti-Nanog polyclonal antibody (1:800, Cell Signaling Technology, Danvers, MA), rabbit anti-Oct4 polyclonal antibody (1:100), mouse anti-p63 monoclonal antibody (1:25, both from Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-K14 polyclonal antibody (1:100), mouse anti-K18 monoclonal antibody (1:100), mouse anti-K10 monoclonal antibody (1:100, all from Lab Vision, Fremont, CA), rabbit anti-Nanog polyclonal antibody (1:800, Cell Signaling Technology), mouse anti-K3 monoclonal antibody (1:100, Millipore, Bedford, MA).

4. Secondary antibodies: Goat anti-mouse IgG$_1$ AlexaFluor 488-conjugated antibody, goat anti-mouse IgG$_2$ AlexaFluor 488-conjugated antibody, donkey anti-rabbit IgG AlexaFluor 594-conjugated antibodies (all from Life Technologies).

5. Hoechst 33342 nuclear staining solution (Sigma, 10 mg/ml stock diluted 1:5,000 in water).

2.3 Flow Cytometry

1. FC fixation buffer: 1 % (v/v) PFA in PBS.

2. Methanol (Fisher) diluted to 90 % (v/v) solution with water.

3. FACS buffer: PBS with 0.5 % (w/v) bovine serum albumin (Sigma), 0.1 % (v/v) Triton X-100 (Fisher), and 0.1 % (w/v) sodium azide (Sigma) added. Store at 4 °C for up to 2 weeks.

4. Primary antibodies: Same as those used for immunofluorescent staining. Mouse and rabbit IgG isotype controls are also recommended for gating negative populations during analysis.

5. Secondary antibodies: Goat anti-mouse IgG$_1$ AlexaFluor 488-conjugated antibody, goat anti-mouse IgG$_2$ AlexaFluor 488-conjugated antibody, donkey anti-rabbit IgG AlexaFluor 633-conjugated antibodies (all from Life Technologies).

3 Methods

Differentiation of hPSCs to epithelial cells involves a stepwise process where simple epithelial cell populations are first derived from hPSC populations. Here, this first step of simple epithelial cell differentiation is initiated using an Src family kinase inhibitor, SU6656, which modulates β-catenin localization (9). The second step in this process involves the maturation of simple epithelial cells to epidermal keratinocyte progenitors, which can then form terminally differentiated epidermal keratinocytes, marked by the expression of several proteins, including cytokeratin 10 (K10).

Throughout this differentiation process, cells can be characterized by monitoring the expression of protein markers for different cell populations at various stages of differentiation. Here, immunofluorescence and flow cytometry methods are described for how to analyze cell populations and confirm successful epithelial differentiation. Overall, the method described here provides an efficient method where epidermal keratinocyte progenitors can be produced from hPSCs via a simple epithelial progenitor that can be expanded indefinitely.

3.1 Cell Growth and Differentiation

1. hPSCs are expanded on a Matrigel-coated substrate in mTeSR1 medium and passaged every 4–5 days. To passage cells, incubate cells in Versene for 3 min to passage cells in colonies or 5 min to passage single cells. Aspirate Versene, and then shear off cells using fresh mTeSR1 medium. Split cells at desired ratio, 1:6 or 1:12. To passage singularized cells, add ROCK inhibitor to fresh mTeSR1 medium. Change medium every day.

2. Begin treatment with hPSC differentiation medium 1 when the cells reach 70–80 % confluence (~100–150k cells/cm^2) by adding 2.5 ml/well for 6-well plates. Change medium daily for 3 days. Some cell death will occur during these 3 days, and a 20–40 % loss of cells should be expected.

3. On the fourth day, add 2 ml/well fresh K-DSFM for a 6-well plate. Change the medium daily until cells reach 80–90 % confluence (~80–120k cells/cm^2). Cells should acquire a simple epithelial morphology (Fig. 1a). At this point, these cells have lost expression of pluripotency markers such as Oct4 and Nanog and have acquired expression of cytoplasmic K18 (Fig. 1c).
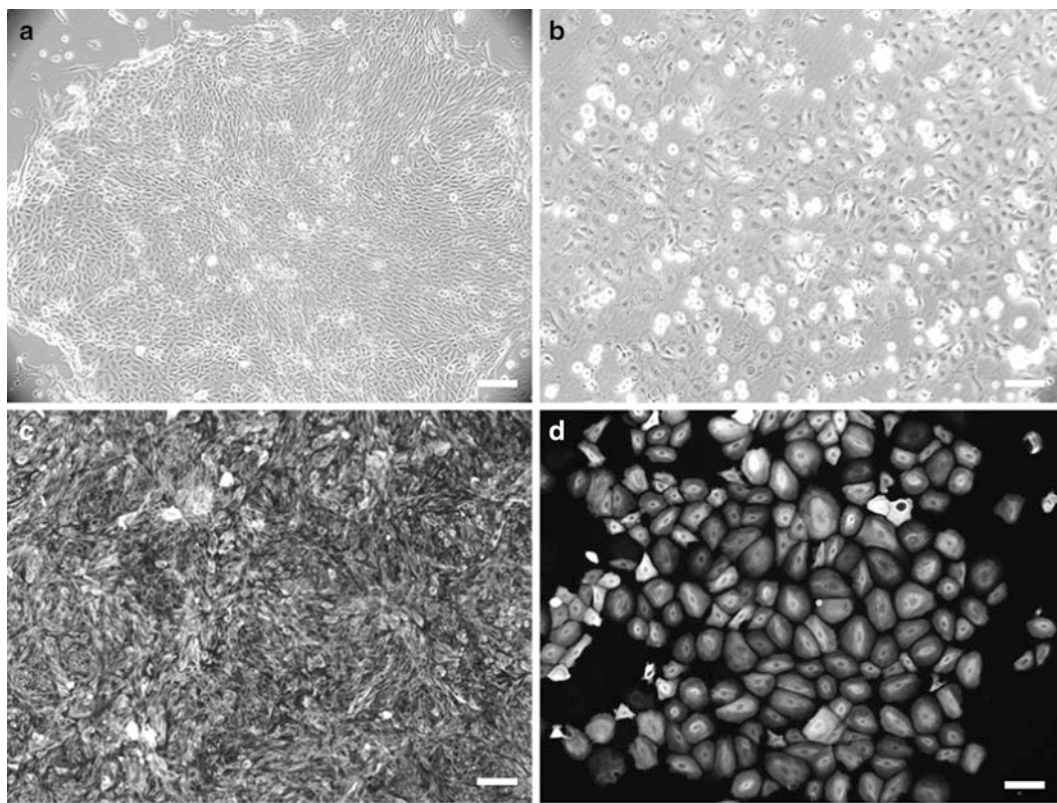
Fig. 1 Images of epithelial cell populations derived from the H9 human embryonic stem cell line using an Src family kinase inhibitor, Su6656. (**a**, **b**) Phase-contrast images of (**a**) simple epithelial cell populations and (**b**) epidermal keratinocyte progenitor populations. (**c**, **d**) Immunofluorescence images demonstrating (**c**) homogeneous expression of K18 throughout the simple epithelial cell population and (**d**) K14 homogeneously expressed throughout the epidermal keratinocyte population. Scale bars in all panels are 100 μm

4. Passage simple epithelial cells by aspirating medium and incubating cells with 1 ml/well of Accutase at 37 °C for 10 min. Quench Accutase using 2 ml/well epithelial cell expansion medium. Pipet to shear off any remaining adhered cells. Split at a ratio of 1:3. Epithelial cells can be plated on either Matrigel or gelatin-coated substrates. When passaging cells to new substrate, resuspend cells in 2 ml/well epithelial expansion medium with ROCK inhibitor.

5. Change epithelial cell expansion medium every other day. Simple epithelial cells can be passaged for >10 passages in this state. Cells will maintain their expression of K18, and cell populations will remain highly enriched (>90 %) in K18-expressing cells (Fig. 2a).

6. To further differentiate simple epithelial cells to epidermal keratinocyte progenitors, aspirate medium and add 2 ml/well of hPSC differentiation medium 2 when the cells reach at least
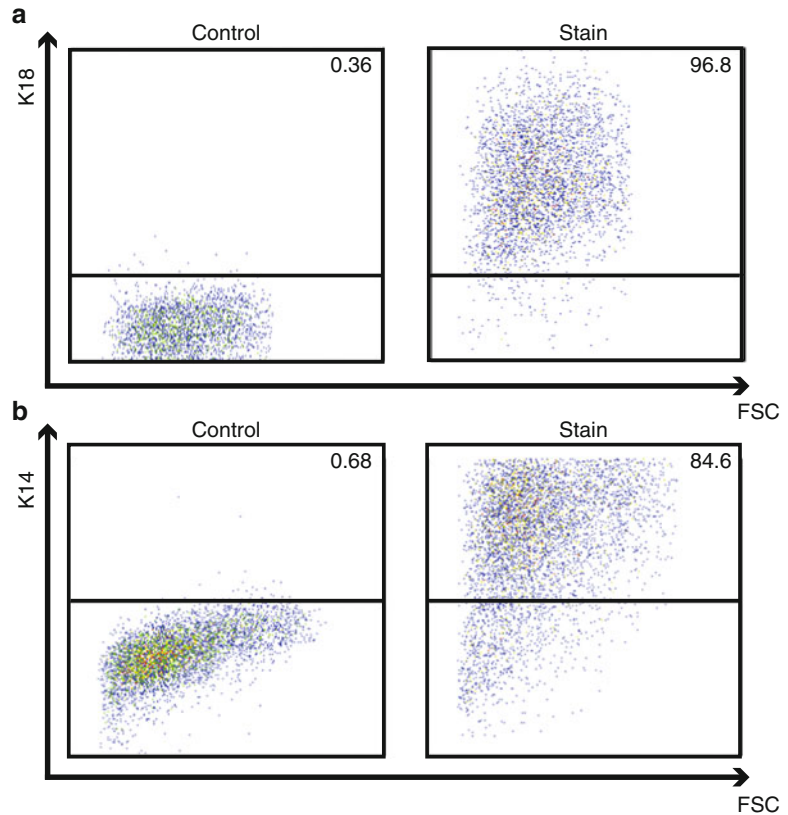
Fig. 2 Representative flow cytometry plots of epithelial cell populations derived from the H9 human embryonic stem cell line using an Src family kinase inhibitor, Su6656. (**a**) Plots demonstrating highly enriched populations of K18-expressing simple epithelial cells and (**b**) highly enriched populations of K14-expressing epidermal keratinocyte progenitor cells. Panels show plots of fluorescence versus forward scatter for both isotype controls and K18 or K14 stains. Percentage of positive cells is indicated in the *upper right corner* of each panel

50 % confluence (~40–50 cells/cm^2). Change the medium daily for 4 days. On the fifth day, change the medium to 2 ml/well of epithelial expansion medium.

7. Change the epithelial expansion medium every other day and expand until the desired number of cells is obtained. These cells obtain a slightly different cobblestone morphology (Fig. 1b) and should express nuclear p63 and cytoplasmic K14 (Fig. 1d). Cell populations at this stage will remain highly enriched (>80 %) in K14-expressing cells (Fig. 2b). Occasionally, <5 % cells can be found in culture expressing corneal marker, K3.

8. These K14+ cells can be terminally differentiated into epidermal keratinocytes using one of several previously reported protocols or assays (5, 6).

3.2 Immuno-fluorescent Staining

1. Aspirate cell culture medium and rinse once with 2 ml/well of PBS for a 6-well plate.

2. Fix cells in 2 ml/well of IF fixation buffer for 15–20 min at room temperature (RT) on a shaker.

3. Rinse cells 2× with 2 ml/well of PBS, and incubate cells in 1 ml/well of blocking buffer for 2 h at RT.

4. Incubate cells with primary antibodies in 300 µl of fresh blocking buffer at the appropriate dilution overnight on a shaker at 4 °C.

5. Rinse cells 3–5× with 2 ml/well of PBS.

6. Incubate cells with secondary antibodies at a dilution of 1:1,000 in 1 ml/well of blocking buffer for 1 h at RT on shaker in the dark.

7. Rinse cells 2× with 2 ml/well of PBS.

8. Incubate cells with 1 ml/well nuclear staining solution for 5 min at RT.

9. Rinse cells 2× with PBS and view on epifluorescent microscope, using a mercury lamp and dichroic filters to excite AlexaFluor 488 and 594 dyes as well as the Hoechst nuclear stain.

3.3 Flow Cytometry

1. Aspirate cell culture medium and rinse once with 2 ml/well of PBS for a 6-well plate.

2. Incubate cells with 1 ml/well of trypsin–EDTA for 5–10 min at 37 °C.

3. Mechanically dissociate larger cell aggregates by pipetting in the trypsin–EDTA solution, and inactivate trypsin with an equal volume of any medium containing at least 20 % FBS.

4. Centrifuge samples at $200 \times g$ for 5 min, and aspirate supernatant.

5. Resuspend cell pellet in 1 ml FC fixation buffer and incubate at 37 °C for 10 min.

6. Centrifuge samples at $200 \times g$ for 5 min and decant supernatant.

7. Resuspend pellet in 1 ml ice-cold methanol solution and hold on ice for 30 min or store at 4 °C for up to 2 weeks.

8. Aliquot 1–2×10^5 cells per sample, and add 2 ml FACS buffer to each sample.

9. Centrifuge cells, and decant supernatant.

10. Add 2 ml FACS buffer to each sample, and repeat centrifugation.

11. Decant supernatant leaving ~50 µl in each sample.

12. Pre-dilute primary antibodies (0.5 μl per sample) in 50 μl of FACS buffer, and add primary antibody solution to each sample. Pipet to resuspend pellet. Incubate samples at 4 °C overnight. Include control samples for each biological sample lacking primary antibodies in each combination for sample compensation.

13. Add 2 ml FACS buffer to each sample, centrifuge, and decant supernatant.

14. Pre-dilute secondary antibodies (0.1 μl per sample) in 50 μl of FACS buffer, and add secondary antibody solution to each sample. Pipet to resuspend pellet. Incubate for 30–60 min at RT in the dark.

15. Add 2 ml FACS buffer to each sample, centrifuge, and decant supernatant.

16. Resuspend each sample in 300 μl FACS buffer and hold on ice or at 4 °C until analysis.

17. Analyze samples on a BD FACSCalibur, LSRII, or equivalent cytometer capable of excitation at 488 and 633 nm. Appropriate cell populations should be gated under forward scatter vs. side scatter plots to exclude debris from analysis. Isotype controls should be used to gate "negative" populations. 488 and 633 fluorophores used for the same samples do not have significant spectral overlap, but fluorescence compensation should be applied for good practice.

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Dual-SMAD Inhibition/WNT Activation-Based Methods to Induce Neural Crest and Derivatives from Human Pluripotent Stem Cells

Stuart M. Chambers, Yvonne Mica, Gabsang Lee, Lorenz Studer, and Mark J. Tomishima

Abstract

The neural crest (NC) is a transient population of multipotent cells giving rise to the peripheral nervous system, skin pigmentation, heart, and facial mesenchyme. The broad cell fate potential of NC makes it an attractive cell fate to derive from human pluripotent stem cells (hPSCs) for exploring embryonic development, modeling disease, and generating cells for transplantation. Here, we discuss recent publications and methods for efficiently differentiating hPSCs into NC. We also provide methods to direct NC into two different terminal fates: melanocytes and sensory neurons.

Keywords Human pluripotent stem cells, Peripheral sensory neurons, Melanocyte, Neural crest, Disease modeling, Dual SMAD inhibition

1 Introduction

The neural crest (NC) is a transient population of multipotent stem cells that emerges from surface ectoderm between the neural tube and nonneural ectoderm during embryonic development and migrates widely throughout the developing embryo. NC arose evolutionarily with early vertebrates and contributes to a variety of tissues including craniofacial cartilage, bone and connective tissue, neurons and glia of the peripheral nervous system, melanocytes (the pigment manufacturing cells of the skin), and smooth muscle in the cardiovascular system. Neurocristopathies are diseases resulting from abnormal NC development (1). Due to their common NC origin, they share overlapping symptoms that can include dysautonomia, insensitivity to pain, loss of pigmentation, heart abnormalities, cleft palate, and neuroendocrine dysfunction to various degrees depending on the neurocristopathy in question. The broad cell fate potential and widespread contribution to various tissues make NC an important cell fate to derive from human pluripotent stem cells (hPSCs) for applications such as studying normal and abnormal (neurocristopathies) human development

and to generate tissue for cell replacement therapy. In fact, recent efforts to model NC diseases using induced pluripotent stem cell (iPSC) technologies have already contributed to understanding basic NC biology and in drug discovery (2–7).

Directing the differentiation of hPSCs is almost never perfect. NC cells were shown to be present in early attempts to direct differentiation into central nervous system (CNS) cultures (3,8). One of the primary methods to produce such CNS cultures is to coculture hPSCs with bone marrow stromal cell lines such as PA-6 (9) or MS5 (10) in the absence of FGF2. Under these conditions, the NC spontaneously arises and can be isolated from the periphery (3) of a CNS neural rosette (11,12). Lawlor and colleagues demonstrated that NC could be isolated from bone marrow cocultures before rosette formation (8). More recently, it was shown that NC and derivatives can be made efficiently without rosettes or coculture with bone marrow stromal cells (2,6,7). How these protocol variations impact the resultant NC remains to be determined: it is possible that the NC (or its derivatives) from these divergent protocols exhibit distinct multilineage potential and marker expression. Recent work from the Studer lab has demonstrated a direct method to make NC from rosettes without stromal cell coculture (Zeltner et al. in preparation for JoVE). These new methods should help to clarify how these protocol changes impact the biology of the resultant NC.

In this chapter, we provide three different protocols for directing NC differentiation from hPSCs without rosettes or coculture. Converting hPSCs to neurectoderm can be accomplished by blocking both BMP and Activin/Nodal/TGFβ branches of SMAD signaling (13), a process termed dual SMAD inhibition (DSi). In the original DSi work, most cells were CNS but NC cells were detected, and the proportion of NC cells could be increased by lowering the cell density at the initiation of neural differentiation. The protocols in this chapter build on this observation by markedly increasing NC yield with DSi through early WNT activation (40–80 % SOX10+ in (7,13) or up to ~90 % p75+/HNK1+ in (6)) using recombinant WNT3 (6,7) or the GSK-3β inhibitors BIO and CHIR 99021 (2,6,7).

Protocol 1 is a simple, DSi/WNT activation-based protocol that creates naïve anterior NC (7). We also describe a variation that posteriorizes NC by adding retinoic acid or FGF2 (7). The anterior NC cells do not express HOX genes but the addition of FGF2 or RA increases HOXB2, B4 and B7 expression by 10- to 1,000-fold over control. HOX gene expression is maintained in melanocytes derived from posteriorized NC. So far, only melanocytes were made from such posteriorized NC: it will be interesting to explore other derivatives from such cultures.

Protocol 2 is a variation and extension of *Protocol 1* that includes factors needed to enhance melanoblast production from the NC (7). It also includes conditions needed to further mature melanoblasts into melanocytes from the NC intermediate.

Protocol 3 rapidly produces sensory neurons (2). Like the first two protocols, it uses DSi and early WNT activation to create SOX10+ NC. But it includes two additional compounds that rapidly induce differentiated sensory neurons from the NC: SU5402 and DAPT are also included in the differentiation beginning at day 2. SU5402 inhibits VEGF, FGF, and PDGF tyrosine kinase signaling (14), and DAPT is a gamma secretase inhibitor that blocks Notch signaling among other pathways dependent on gamma secretase cleavage for signal propagation (15).

2 Materials

2.1 hPSC Culture

1. Human embryonic stem cells or induced pluripotent stem cells (*see* **Note 1**).

2. MEF CF-1 mitomycin-C treated mouse embryonic fibroblasts (MEFs; Applied StemCell, Inc., ASF-1223).

3. MEF medium containing 900 mL of Dulbecco's modified Eagle medium (Life Technologies, cat. no. 11965-118), 100 mL fetal bovine serum (Life Technologies, cat. no. 26140-095). The medium is sterile-filtered before use.

4. Recombinant FGF2 (R&D, cat. no. 233-FB-001MG/CF) prepared as 100 μg/mL stock in 1× DPBS containing 0.1 % BSA (*see* **Note 2** about growth factor preparation and use).

5. hESC medium containing 800 mL of DMEM/F12 hESC medium (Life Technologies, cat. no. 11330-032), 200 mL knockout serum replacement (KSR; Life Technologies, cat. no. 10828-028), 5 mL L-glutamine (200 mM, Life Technologies, cat. no. 25030-081), 10 mL MEM non-essential amino acids (MEM NEAA; Life Technologies, cat. no. 11140-050), and 1 mL of 2-mercaptoethanol (Life Technologies, cat. no. 21985-023). The medium is sterile-filtered in a hood and 60 μl of FGF2 stock is added after filtration to a final concentration of 6 ng/mL.

6. Sterile 1× DPBS (Gibco/Life Technologies, cat. no. 14190).

7. Gelatin-coated dishes made by adding enough gelatin to coat the bottom of the dish (0.1 % gelatin in PBS, Chemicon/Millipore, cat. no. ES-006-B). Allow gelatin to incubate for at least 15 min prior to plating cells. Remove gelatin before plating.

8. Dispase (5 U/mL, Stem Cell Technologies, cat. no. 07913).

9. MEF conditioned medium (CM) is harvested from MEF-coated flasks. MEFs are plated at a density of 50,000 cells/cm² in a T225 flask in MEF medium. The next day, the cells are washed once with PBS before adding 100 mL of hESC medium. Incubate medium with MEFs for 24 h before removal. The medium is now known as "conditioned medium" (CM) and can be directly used or stored at 4 °C for less than 2 weeks. Additional hESC medium can be conditioned daily for up to 10 days on the same flask of feeders. Just before using, FGF2 is added to CM to a final concentration of 10 ng/mL, hereafter called complete CM (cCM).

2.2 Naïve Neural Crest Differentiation

1. Accutase (Innovative Cell Technologies, cat. no. AT104).

2. Nylon mesh cell strainers, 40 μm pore size (BD Falcon, cat. no. 352340).

3. Matrigel Basement Membrane Matrix (BD Bioscience; cat. no. 354234: we only use lots that contain over 10 mg/mL protein). Thaw the frozen vial of Matrigel on ice overnight in a 4 °C refrigerator. Prepare 1 mL aliquots in a 50 mL centrifuge tube using chilled pipettes and freeze at -20 °C. Matrigel must be thawed slowly to prevent gelatinization. Chilled pipettes and 50 mL centrifuge tubes should be used when making aliquots of the Matrigel.

4. KSR medium containing 820 mL of Knockout DMEM (Life Technologies; cat. no. 10829-018), 150 mL KSR (Life Technologies, cat. no. 10828-028), 10 mL L-glutamine (200 mM, Life Technologies, cat. no. 25030-081), 10 mL MEM NEAA (Life Technologies, cat. no. 11140-050), and 1 mL of 2-mercaptoethanol (Life Technologies, cat. no. 21985-023).

5. N2 medium containing DMEM/F12 powder (Gibco/Life Technologies, cat no. 12500-062) in 550 mL of distilled water. 1.55 g of glucose (Sigma, cat. no. G7021), 2.00 g of sodium bicarbonate (Sigma, cat. no. S5761), putrescine (100 μL aliquot of 1.61 g prepared in 100 mL of distilled water; Sigma, cat. no. P5780), progesterone (20 μL aliquot of 0.032 g prepared in 100 mL 100 % ethanol; Sigma, cat. no. P8783), sodium selenite (60 μL aliquot of 0.5 mM solution in distilled water; Bioshop Canada, cat. no. SEL888), and 100 mg of transferrin (Celliance/Millipore, cat. no. 4452-01) are added. 25 mg of powdered insulin (Sigma, cat. no. I6634) is added to 10 mL of 5 mM NaOH and is shaken until completely prepared. The solubilized insulin is added to the medium, and double-distilled water (with a resistance of 18.2 MΩ) is added to a final volume of 1,000 mL before sterile filtration.

6. SB-431542 (Tocris Bioscience, cat. no. 1614) prepared as 10 mM stock in 100 % ethanol (1,000× stock).

7. LDN-193189 (Stemgent, cat. no. 04-0074) prepared as 5 mM stock in DMSO (5,000× stock).

8. Y-27632 dihydrochloride (Tocris Bioscience, cat. no. 1254) prepared as 10 mM stock in sterile water (1,000× stock).

9. CHIR 99021 (Tocris Bioscience, cat. no. 4423) prepared as 3 mM stock in DMSO (1,000× stock).

2.3 Melanocyte Differentiation

1. Poly-L-ornithine hydrobromide (Sigma-Aldrich, cat. no. P3655), prepared as 15 mg/mL stock in sterile water (1,000× stock).

2. 1 mg/mL mouse laminin I solution (Cultrex, R&D Systems, cat. no. 3400-010-1, 1,000× stock).

3. BMP4 (R&D Systems, cat no. 314-bp), prepared as a 10 μg/mL stock in 1× DPBS containing 0.1 % BSA (1,000× stock).

4. EDN3 (American Peptide Company, cat. No. 88-5-10B) prepared as a 100 μM stock in 1x DPBS containing 0.1 % BSA (1,000× stock).

5. dbcAMP (Sigma cat. no. D-0260 or EMD4biosciences cat. no. 28745) prepared as a 100 mM stock solution in 1× DPBS containing 0.1 % BSA (200× stock).

6. Fibronectin (FN; Becton Dickinson cat. no. 356008) prepared as 1 mg/mL stock solution in 0.1 % BSA in PBS (500× stock).

7. Stem Cell Factor (SCF; Peprotech cat. no. 300-07) prepared as 50 mg/mL stock solution in 1× DPBS containing 0.1 % BSA (1,000× stock).

8. NB/Mel medium: To Neurobasal medium (Life Technologies cat. no. 21103049) add 2 % B27 supplement (Life Technologies cat. no. 12587010), 2 % N2 supplement (Life Technologies cat. no. 17502048), 3 μM CHIR 99021, 10 ng/mL FGF2, 500 μM dbcAMP, 25 ng/mL BMP4, 100 nM EDN3, and 50 ng/mL SCF.

9. PO/LAM/FN-coated dishes: Add 15 μg/mL PO (in PBS) to cell culture dish and incubate overnight. The next day, aspirate the PO solution, wash the coated dish three times with PBS and then add 1 μg/mL laminin and 10 μg/mL fibronectin (in PBS). Incubate at least 2 h, preferably overnight—longer incubation improves adhesion. Before using plates, aspirate medium and, if necessary, dry the plates by running an aspirating pipette just over the surface of the plate without touching the PO/LAM/FN film.

2.4 Sensory Neuron Differentiation

1. Accutase (Innovative Cell Technologies, cat. no. AT104).

2. Nylon mesh cell strainers, 45 μm pore size (BD Falcon, cat. no. 352340).

3. Matrigel Basement Membrane Matrix (BD Bioscience; cat. no. 354234: we only use lots that contain over 10 mg/mL protein). Thaw the frozen vial of Matrigel on ice overnight in a 4 °C refrigerator. Prepare 1 mL aliquots in a 50 mL centrifuge tube using chilled pipettes and freeze at −20 °C. Matrigel must be thawed slowly to prevent gelatinization. Chilled pipettes and 50 mL centrifuge tubes should be used when making aliquots of the Matrigel.

4. KSR medium containing 820 mL of Knockout DMEM (Life Technologies; cat. no. 10829-018), 150 mL KSR (Life Technologies, cat. no. 10828-028), 10 mL L-glutamine (200 mM, Life Technologies, cat. no. 25030-081), 10 mL MEM NEAA (Life Technologies, cat. no. 11140-050), and 1 mL of 2-mercaptoethanol (Life Technologies, cat. no. 21985-023).

5. N2 medium containing DMEM/F12 powder (Gibco/Life Technologies, cat no. 12500-062) in 550 mL of distilled water. 1.55 g of glucose (Sigma, cat. no. G7021), 2.00 g of sodium bicarbonate (Sigma, cat. no. S5761), putrescine (100 μL aliquot of 1.61 g prepared in 100 mL of distilled water; Sigma, cat. no. P5780), progesterone (20 μL aliquot of 0.032 g prepared in 100 mL 100 % ethanol; Sigma, cat. no. P8783), sodium selenite (60 μL aliquot of 0.5 mM solution prepared in distilled water; Bioshop Canada, cat. no. SEL888), and 100 mg of transferrin (Celliance/Millipore, cat. no. 4452-01) are added. 25 mg of powdered insulin (Sigma, cat. no. I6634) is added to 10 mL of 5 mM NaOH and is shaken until completely dissolved. The solubilized insulin is added to the medium, and double-distilled water (with a resistance of 18.2 MΩ) is added to a final volume of 1,000 mL before sterile filtration.

6. SB-431542 (Tocris Bioscience, cat. no. 1614) prepared at 10 mM in 100 % ethanol (1,000× stock).

7. LDN-193189 (Stemgent, cat. no. 04-0074) prepared at 5 mM in DMSO (5,000× stock).

8. Y-27632 dihydrochloride (Tocris Bioscience, cat. no. 1254) prepared at 10 mM in sterile water (1,000× stock).

9. CHIR 99021 (Tocris Bioscience, cat. no. 4423) prepared at 3 mM in DMSO (1,000× stock).

10. SU5402 (Biovision, cat no. 1645-1) prepared at 5 mM in DMSO (1000× stock).

11. DAPT (R&D Systems, cat. no. 2634) prepared at 10 mM in DMSO (1000× stock.)

12. Recombinant Human BDNF (R&D Systems, cat. no. 248-BD) prepared at 10 μg/mL in 1× DPBS containing 0.1 % BSA (500× stock).

13. Recombinant human GDNF (PeproTech, cat. no. 450-10) prepared at 10 μg/mL in 1× DPBS containing 0.1 % BSA (500× stock).

14. Recombinant human beta-NGF (PeproTech, cat. no. 450-01) prepared at 25 μg/mL in 1× DPBS containing 0.1 % BSA (500× stock).

15. Poly-L-ornithine hydrobromide (PO; Sigma-Aldrich, cat. no. P3655) prepared at 15 mg/mL in sterile water (1000× stock).

16. 1 mg/mL mouse laminin I solution (LAM; Cultrex, R&D Systems, cat. no. 3400-010-1, 1000× stock).

17. PO/LAM-coated dishes: Add 15 μg/mL PO (in PBS) to cell culture dish and incubate overnight. The next day, aspirate the PO solution, wash the coated dish three times with PBS and then add 1 μg/mL LAM (in PBS). Incubate at least 2 h, preferably overnight—longer incubation improves adhesion. Before using plates, aspirate medium and, if necessary, dry the plates by running an aspirating pipette just over the surface of the plate without touching the PO/LAM film.

3 Methods

3.1 Grooming and Preparation of hPSCs for Differentiation

1. Groom pluripotent cells (hESCs or hiPSCs) using the picking hood, objective microscope and 200 μL pipette with sterile filter tips. Remove any pluripotent colonies that have the appearance of differentiated cells, irregular borders, or transparent centers.

2. Aspirate hESC medium and add minimal Accutase to coat the dish and let sit at 37 °C until all colonies are rendered to single cells (approximately 30 min). Amount used: 1 mL for a 6-well dish, 2 mL for a 6 cm dish, or 5 mL for a 10 cm dish.

3. Avoiding bubbles, triturate the cells in the dish using a pipette containing additional hESC medium.

4. Pipette hESC medium until there is a single cell suspension and filter using a 40 μm nylon cell strainer to remove clumps.

5. Wash and centrifuge cells (200 × g for 5 min) twice in hESC medium to remove all traces of Accutase.

6. While washing, prepare Matrigel-coated dishes. Add 19 mL of DMEM:F12 to the Matrigel aliquot thawed overnight on ice (*see* Sect. 2.2) and pipette until homogenous. Work quickly and do not let the Matrigel warm up or it will polymerize. A 40 μm nylon cell strainer can be used to remove any insoluble clumps. Coat culture dishes with the diluted Matrigel solution and incubate for 1 h in the hood.
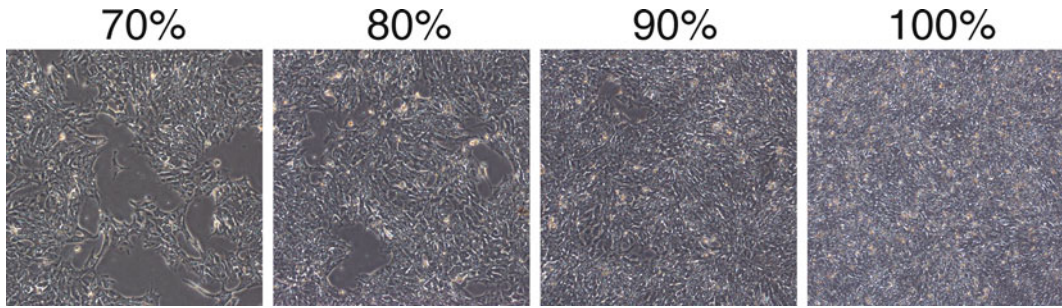
Fig. 1 The density of hPSCs during neural induction affects cell fate. A range of cell densities is shown here for comparison. With kind permission from Springer Science + Business Media: Methods in Molecular Biology, Neurodegeneration: Methods and Protocols, Chapter 6, Converting Human Pluripotent Stem Cells to Neural Tissue and Neurons to Model Neurodegeneration, 793 (2011): 87–97, Chambers SM, Mica Y, Studer L, Tomishima MJ, Figure 1

7. After washing hPSCs, resuspend the cells in hESC medium containing 10 μM Y-27632 and plate on a gelatin-coated dish of the same size as step 2 (e.g. 10 cm gelatin-coated dish for a 10 cm dish of Accutase treated cells). (*Y-27632 prevents single hPSCs from dying* (16).)

8. Incubate dish at 37 °C for 30 min in a cell incubator.

9. After 30 min, collect the nonadherent cells and wash the dish with hESC medium containing 10 μM Y-27632 and centrifuge cells.

10. Resuspend the cells in cCM + 10 μM Y-27632.

11. Determine the cell concentration using a hemocytometer and add cCM+ 10 μM Y-27632 to the appropriate cell volume to achieve 10,000–20,000 cells/cm^2.

12. Aspirate Matrigel solution and rinse culture dishes once with DMEM:F12 prior to plating cells.

13. Plate pluripotent cells on Matrigel-coated dishes at 10,000–20,000 cells/cm^2.

14. Twenty-four hours after plating, aspirate medium and add fresh cCM+ 10 μM Y-27632.

15. Forty-eight hours after plating, aspirate the medium and add fresh cCM—from this point on, Y-27632 is no longer necessary. Cells can be maintained for additional days in cCM until the ideal differentiation density is obtained (~60–80 % confluence for neural crest; *see* Fig. 1 for examples).

3.2   Protocol 1: Naïve Neural Crest

The naïve neural crest protocol uses a combination of DSi and early WNT activation to make anterior neural crest that can be sorted at day 11 to make a pure population of cells. To posteriorize neural crest cultures, *see* **Note 6**.

1. Initiate differentiation when the cells are 60–80 % confluent (*day 0*). To initiate differentiation, replace the medium with KSR medium containing 10 μM SB-431542 and 500 nM LDN-193189 (*see* **Note 3**).

2. On *day 1* of the differentiation, aspirate medium and replace with KSR medium containing 10 μM SB-431542 and 500 nM LDN-193189.

3. On *day 2* of the differentiation, aspirate medium and replace with KSR medium containing 10 μM SB-431542, 500 nM LDN-193189, and 3 μM CHIR 99021 (*activates WNT signaling*).

4. On *day 3* of the differentiation, aspirate medium and replace with KSR medium containing 10 μM SB-431542 and 3 μM CHIR 99021 (*release BMP signaling to endogenous levels by omitting LDN*).

5. On *day 4* of the differentiation, aspirate medium and replace with 75 % KSR, 25 % N2 medium containing 3 μM CHIR 99021 (*begin transition to neural medium; see* **Note 4**).

6. On *day 6* of the differentiation, aspirate medium and replace with 50 % KSR, 50 % N2 medium containing 3 μM CHIR 99021.

7. On *day 8* of the differentiation, aspirate medium and replace with 25 % KSR medium, 75 % N2 medium containing 3 μM CHIR 99021.

8. On *day 10* of the differentiation, aspirate medium and replace with N2 medium containing 3 μM CHIR99021.

9. On *day 11* your cells are ready to replate. To make pure neural crest, sort for HNK1+ and p75+ cells (*see* **Notes 5** and **6**).

3.3 Protocol 2: Melanocyte Induction

The melanocyte induction protocol is nearly the same as the naïve neural crest protocol above except that it includes Endothelin-3 (EDN3) and BMP-4. It does require a sort at day 11 since the melanocyte induction medium is selective and eliminates other cell fates on its own.

1. Prepare hPSCs for neural induction as described above in Sect. 3.1 (Grooming and preparation of hPSCs for differentiation).

2. Initiate differentiation when the cells are 60–80 % confluent (*day 0*). To initiate differentiation, aspirate medium and replace with KSR medium containing 10 μM SB-431542 and 500 nM LDN-193189.

3. On *day 1* of the differentiation, aspirate medium and replace with KSR medium containing 10 μM SB-431542 and 500 nM LDN-193189.

4. On *day 2* of the differentiation, aspirate medium and replace with KSR medium containing 10 μM SB-431542, 500 nM LDN-193189, and 3 μM CHIR 99021 (*activates WNT signaling*).

5. On *day 3* of the differentiation, aspirate medium and replace with KSR medium containing 10 μM SB-431542 and 3 μM CHIR 99021 (*release BMP signaling to endogenous levels by omitting LDN*).

6. On *day 4* of the differentiation, aspirate medium and replace with 75 % KSR, 25 % N2 medium containing 3 μM CHIR 99021 (*begin transition to neural medium*; *see* **Note 4**).

7. On *day 6* of the differentiation, aspirate medium and replace with 50 % KSR, 50 % N2 medium containing 3 μM CHIR 99021, 25 ng/mL BMP-4, and 100 nM endothelin-3 (EDN3).

8. On *day 8* of the differentiation, aspirate medium and replace with 25 % KSR medium, 75 % N2 medium containing 3 μM CHIR 99021, 25 ng/mL BMP4, and 100 nM EDN3.

9. On *day 10* of the differentiation, aspirate medium and replace with N2 medium containing 3 μM CHIR 99021, 25 ng/mL BMP-4, and 100 nM EDN3.

10. On *day 11*, the cells are replated. Cells are dissociated with Accutase for 30 min and washed twice with plain Neurobasal medium before resuspension in NB/Mel medium (see Sect. 2.3 for medium preparation) at a concentration of 2×10^6 cells/mL and plated on dried poly-ornithine-, laminin-, and fibronectin-coated plates (see Sect. 2.3 for dish preparation) as 10 μl droplets. If the plate has been sufficiently dried, the droplets should be remain as a well-defined meniscus and not run. Let stand in the hood for 20 min before slowly adding more NB/Mel medium.

11. After plating melanocyte progenitors as droplets, continue feeding with NB/Mel every 2–3 days (*see* **Note 7**).

12. Cells should be passaged once a week at a ratio of ~1:6 (plating as droplets is no longer necessary at this stage). Use Accutase to dissociate cells and wash twice with plain Neurobasal medium before replating in NB/Mel medium. Cells should continue to be maintained on PO/LAM/FN plates; however, the plates no longer need to be dried before use (*see* **Note 8**).

13. *Optional*: Melanocytes can be cryopreserved using 5 % DMSO and 95 % NB/Mel medium. To enhance recovery after cryo-preservation, the initial plating of melanocytes can be at an ultra-high density (2×10^6 cells/mL, 10 μl droplets) on dried PO/LAM/FN-coated plates before maintenance as above.

3.4   Protocol 3: Sensory Neuron Induction

Sensory neuron induction includes the basic DSi protocol with early WNT activation to increase NC similar to the previous two protocols. It differs from the previous protocols because it includes two additional small molecules (DAPT and SU5402) to promote neuron differentiation over proliferation. It is complete around day 12 when neuronal processes project away from neural crest progenitor domains in the dish. For best survival, neurons are passaged onto either Matrigel or PO/LAM treated plates prior to day 15 for extended culture in N2 medium containing the neurotrophins BDNF, GDNF, and NGF. A mitotic block such as mitomycin-c is recommended for long-term culturing since some proliferative cells do persist. Cells are normally replated in droplets and optimal plating density may vary from cell line to cell line and should be empirically determined. Sensory neurons can be kept in culture as long as the medium is fresh and mouse laminin I is added to the medium weekly to maintain an adequate substrate for the long-term neurons. The sensory neurons will continue to mature, establish a far-reaching and elaborate axonal network, and begin to express additional sensory ion channels after 100–150 days.

1. Prepare hPSCs for neural induction as described above in Sect. 3.1 (*Grooming and preparation of hPSCs for differentiation*).

2. To initiate differentiation, aspirate the medium and add KSR medium containing 10 μM SB-431542 and 100 nM LDN-193189 (*day 0*). (*initiate neural induction via DSi*).

3. On *day 1* of differentiation, aspirate the medium and add KSR medium containing 10 μM SB-431542 and 100 nM LDN-193189.

4. On *day 2* of differentiation, aspirate the medium and add KSR medium containing 10 μM SB-431542, 100 nM LDN-193189, 3 μM CHIR 99021, 10 μM DAPT, and 5 μM SU5402. (*activate WNT while blocking gamma secretase/Notch, VEGF/FGF/PDGF signaling*).

5. On *day 4* of differentiation, aspirate the medium and add KSR/N2 (3:1) medium containing 10 μM SB-431542, 100 nM LDN-193189, 3 μM CHIR 99021, 10 μM DAPT, and 5 μM SU5402 (*final concentrations are for the combined KSR/N2 mixture*).

6. On *day 6* of differentiation, aspirate the medium and add KSR/N2 (1:1) medium containing 3 μM CHIR 99021, 10 μM DAPT, and 5 μM SU5402. (*release BMP and Activin/Nodal/TGFß signaling to endogenous levels.*)

7. On *day 8* of differentiation, aspirate the medium and add KSR/N2 (1:3) medium containing 3 μM CHIR 99021, 10 μM DAPT, and 5 μM SU5402 (*see* **Note 9**).

8. On *day 10* of differentiation, aspirate the KSR/N2 and add N2 medium containing 3 μM CHIR 99021, 10 μM DAPT, and 5 μM SU5402.

9. On *day 12* of differentiation, cells can be passaged as single cells using Accutase to disassociate onto PO/LAM (*see* Sect. 2.4 for preparation) or Matrigel-coated dishes with N2 medium containing 20 ng/mL BDNF, 20 ng/mL GDNF, and 50 ng/mL NGF. Cells may be spotted as droplets to increase survival.

10. Cells should be fed 2–3 times a week depending on the color of the medium with N2 medium containing 20 ng/mL BDNF, 20 ng/mL GDNF, and 50 ng/mL NGF. Weekly, mouse 1 μg/mL laminin I is added to cultures to maintain neuron adhesion to the dish.

11. *Optional mitomycin C treatment at day 14.* If neurons are being maintained for greater than 3 weeks, the cells can be exposed to mitomycin C to halt mitosis and enrich for neurons on day 14 after differentiation. Aspirate the medium, add N2 medium containing 1 μg/mL of mitomycin C, return the dish to the incubator for 1 h, and replace the medium with fresh N2 medium containing 20 ng/mL BDNF, 20 ng/mL GDNF, and 50 ng/mL NGF. Proliferating cell death is observed between 24 and 48 h after mitomycin C exposure. The process can be repeated if proliferative cells later emerge.

4   Notes

1. Human pluripotent stem cells differ in their properties, so optimization might be required for a cell line of interest. These protocols were primarily derived from H9 hESCs although both studies also demonstrated their utility on hiPSCs. The plating efficiency, the optimal NC-inducing cell density and the concentration and timing of growth factors are all variables that might need adjustment to find the best yield for a given pluripotent stem cell source.

2. We generally purchase growth factors in bulk as lyophilized protein. Most factors are reconstituted in 1x DPBS containing 0.1 % BSA at a concentration recommended by the manufacturer: storing recombinant proteins at too low of a concentration promotes instability. We do not sterile-filter recombinant proteins because proteins can adhere to the membrane, lowering the effective concentration of the preparation. Single-use aliquots are stored at −80 °C. After thawing, we store aliquots at 4 °C for up to 2 weeks.

3. Cell density when initiating differentiation determines the relative amounts of CNS versus NC cells produced, with lower confluence biasing toward NC and higher confluence toward CNS. However, cell viability diminishes at very low confluence (<50 % confluent). Initial seeding densities ("plating

efficiency") can vary between pluripotent cell lines and should be empirically determined to reach ideal starting conditions.

4. At this point, it often helps to overfill your dishes/wells with medium. The cells become very confluent and the medium will turn yellow quickly if you do not. It is normal to observe a large number of dead, floating cells at this point of the differentiation.

5. By day 11 you will have obtained your maximum yield of neural crest progenitors. The population contains a mixture of CNS and neural crest populations. Using FACS for a Sox10::GFP reporter, we have determined that neural crest yield ranges from 40 to 80 % of the population.

6. Posteriorizing NC: Mica et al. demonstrate that these cultures can be posteriorized through the addition of either FGF2 or retinoic acid (RA) beginning as early as day 2, although in some conditions this erodes the number of SOX10+ cells. As such, the optimal posteriorizing condition is to add 1 μM RA or 10 ng/mL FGF2 at day 6 (and every feed thereafter until day 11 in this protocol). RA was more potent at inducing HOX genes at these doses but the other properties between these two posterior NC populations were not yet explored.

7. Pigmentation should start to be visible within clusters of cells by the end of the first week (you may have to view the plate over a white background such as a sheet of paper to discern these early small, dark clusters. Cells will become progressively more pigmented over time, until the entire plate is uniformly pigmented.

8. We have found that the NB/Mel medium is best suited for maintaining and expanding hES- and iPS-derived melanocytes in culture for extended periods of time. Cells can be briefly cultured in commercially available M254 medium (Life Technologies cat. no. M-254-500), which further enhances their typical spindle-like melanocyte morphology. However, we have found that this medium does not support the extended culture of our cells.

9. Some toxicity may be observed that is inversely correlated with efficiency of SOX10 induction.

Acknowledgments

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Accelerated Three-Dimensional Neuroepithelium Formation from Human Embryonic Stem Cells and Its Use for Quantitative Differentiation to Human Retinal Pigment Epithelium

Yu Zhu, Sven Schreiter, and Elly M. Tanaka

Abstract

Successful applications of pluripotent stem cells to cell-based therapies will rely on rapid and efficient methods to differentiate cells toward the target cell type. While methods have been developed for the generation of some medically relevant cell types including retinal pigment epithelium (RPE) cells, such protocols are lengthy and result in a heterogeneous cell mixture of RPE and non-RPE cells, requiring manual subselection and expansion. Such considerations have significant limiting impact of therapeutic applicability. Here we describe the accelerated three-dimensional neuroepithelial cyst culture of human embryonic stem cells (hESCs) and its utility to achieve quantitative production of RPE cell sheet with no manual selection in 30 days.

Keywords: Human embryonic stem cells, Three-dimensional, Neuroepithelial cyst, Retinal pigment epithelium

1 Introduction

Differentiation along the neural lineage to obtain different neural cell types is one major endeavor in human embryonic stem cell (hESC) research. Cells are typically differentiated in two-dimensional (2D) culture, passing through a rosette-forming neuroepithelial stage before maturing into later stage neural progenitor cells, or in various embryoid body/floating aggregate suspension cultures that are enriched for neuroepithelial cells before plating to generate 2D rosette cultures, or being maintained in floating culture (1–10).

One specific aspect of neural induction is differentiation of hESCs to retinal tissues including the neural retina and the retinal pigment epithelium (RPE). Here, we particularly focus on RPE which continues to be an important target for hESC differentiation being a well-defined, experimentally accessible portion of the central nervous system. Degeneration of the RPE in diseases such as amacular degeneration leads to photoreceptor loss and blindness.

Two principal types of protocol have been described for forming RPE from hESCs. Selection of spontaneously differentiating colonies in hESC cultures was initially described and takes at least 5–7 weeks post-induction for handpicked pigmented clusters to grow in size and number (11–14). On the other hand, several methods starting with floating aggregates have contributed enormous progress, reporting 10–34 % efficiency in 4–17 weeks. The application of the inductive factors such as Activin A and nicotinamide markedly improved RPE yield, yet selection is still necessary to obtain a uniform RPE cell layer (15–18). Recently, Buchholz et al. developed an efficient 2D culturing protocol using a combination of Dkk1, IGF1, Noggin, and nicotinamide promoting optic fate, followed by inhibiting FGF signaling along with Activin A and VIP treatment to drive RPE differentiation, which generated sheets of RPE progenitors starting to pigment by 2 weeks of differentiation (19). Thus, a protocol that quantitatively induces RPE formation through a developmentally faithful progression would be valuable for studying the development of human RPE on a cell biological and biochemical level. Furthermore, since the ability to expand RPE once it is already formed is limited by the tendency of passaged RPE to become fibroblastic, a method to quantitatively produce RPE directly from hESCs would allow larger amounts of RPE to be produced, a limitation in current RPE protocols.

A current limitation in embryoid body and floating aggregate culture as a starting point for neural differentiation is the heterogeneity, and ill-defined organization of the suspended cells before differentiating to neuroepithelium upon exposure to defined culture conditions or via "self-organization." The in vitro reconstitution of epithelial structure and function has been previously described for kidney and mammary epithelium, using either three-dimensional (3D) culture in collagen I or collagen IV-based matrices, or by 2D culturing on transwell filters that allow full apicobasal polarity to develop across the cells to form a tight epithelium with defined transepithelial resistance (20, 21). Here, by applying culturing techniques for polarized epithelial cells to hESCs we describe a method that directly differentiates cells to epithelial cysts that stably harbor a single lumen within 1 day and uniformly achieves pseudostratified optic vesicle neuroepithelium identity in 5 days. These cysts represent a starting point for reconstituting complex aspects of neural development in response to developmental cues. By further culturing the cysts in 2D epithelial culture for about 2 weeks, we quantitatively differentiate them into a confluent, post-mitotic RPE layer that forms tight junctions with the expected transepithelial resistance of human fetal RPE (22). This protocol bypasses requirements for cell selection or expansion of small RPE colonies when producing RPE, a significant consideration for cell yield and an essential requirement for studying human RPE biogenesis.

2   Materials

2.1   Human Embryonic Stem Cell Maintenance

Prepare all solutions at room temperature and store under indicated conditions.

1. DMEM/F-12.
2. mTeSR™1.
3. mFreSR™.
4. Dispase, 1 U/mL. Make 10 mL aliquots and store at −20 °C.
5. BD Matrigel™ hESC-qualified Matrix. Prepare 100 μL aliquots on ice and store at −80 °C (see Note 1).

2.2   Human Embryonic Stem Cell Differentiation

1. BD Matrigel™ Basement Membrane Matrix. Prepare 300 μL aliquots on ice and store at −80 °C (see Note 1).
2. BD Matrigel™ Basement Membrane Matrix, Growth Factor Reduced. Prepare 50 μL aliquots on ice and store at −80 °C (see Note 1).
3. Cell Recovery Solution.
4. TrypLE™.
5. Soybean Trypsin Inhibitor. Stock concentration: 10 mg/mL. Prepare 100 μL aliquots and store at −20 °C (see Note 2).
6. DMEM/F12, GlutaMax™.
7. DMEM, High Glucose, GlutaMax™, Pyruvate.
8. Knockout™ Serum Replacement. Prepare 50 mL aliquots and store at −20 °C.
9. MEM Non-essential Amino Acids Solution (100×).
10. Neurobasal Medium (1×).
11. B27 Serum-Free Supplement (50×). Prepare 1 mL aliquots and store at −20 °C.
12. Transferrin.
13. Sodium Pyruvate 100 mM Solution.
14. L-Glutamine 200 mM. Prepare 5.5 mL aliquots and store at −20 °C.
15. Penicillin-Streptomycin 10,000 U/mL. Prepare 5 mL aliquots and store at −20 °C.
16. Recombinant Human/Mouse/Rat Activin A. Dissolve the powder in PBS/0.1 % BSA (see Note 3). Stock concentration: 10 μg/mL. Prepare 100 μL aliquots. Flash freeze the aliquots in liquid nitrogen and store at −80 °C.
17. Bovine Serum Albumin (BSA).
18. Insulin.

19. Progesterone.

20. Putrescine.

21. Sodium selenite.

22. β-mercaptoethanol.

2.3 Media Preparation

1. N2-supplement. Add 500 μL 100 mg/mL Transferrin (see Note 4), 335 μl 75 mg/mL BSA solution (see Note 5), 16.5 μL 0.6 mg/mL Progesterone (see Note 6), 50 μL 160 mg/mL Putrescine (see Note 7), 5 μL 3 mM Sodium selenite (see Note 8), and 625 μL 20 mg/mL Insulin (see Note 9) into 3.468 mL DMEM/F12, GlutaMax^{TM} to make a 5 mL N2-supplement. Mix well by pipetting up and down. Prepare 500 μL aliquots of N2-supplement and store at −20 °C (see Note 10).

2. N2B27 medium. Mix 50 ml DMEM/F12, GlutaMax^{TM}, 50 ml Neurobasal Medium, 1 ml B27 Serum-Free Supplement, 0.5 ml N2-supplement, 100 μL β-mercaptoethanol solution (see Note 11), 250 μL Pyruvate + Glutamine solution (see Note 12), and 1 mL Penicillin-Streptomycin. The complete N2B27 medium should be sterile filtered through a 0.22 μm filter system and kept at 4 °C for up to 1 week before use.

3. RPE medium. Mix 200 mL DMEM (High Glucose, GlutaMax^{TM}, Pyruvate), 50 mL Knockout^{TM} Serum Replacement, 2.5 mL MEM Non-essential Amino Acids Solution, and 1.25 mL Glutamine + β-mercaptoethanol solution (see Note 13). The complete RPE medium should be sterile filtered through a 0.22 μm filter system, aliquoted, and stored at −20 °C. Thawed RPE medium should be stored at 4 °C for up to 2 weeks before use.

3 Methods

3.1 Maintenance of hESCs

Culture hESC lines H9 and H1 in mTeSR^{TM}1 medium on BD Matrigel^{TM} hESC-qualified Matrix-coated 6-well plate (see Note 14). For storage, the hESC colonies should be suspended in mFreSR^{TM} (see Note 15), frozen in an isopropanol freezing container at −80 °C overnight and transferred into liquid nitrogen the next day.

hESCs are ready to passage when the colonies are large, beginning to merge, and have centers that are dense and phase-bright compared to their edges (see Note 16). The detailed protocol for passaging is shown below:

1. Warm mTeSR^{TM}1 medium (see Note 17), aliquoted Dispase, and DMEM/F-12 in a 25 °C water bath. Coat a 6-well plate with BD Matrigel hESC-qualified matrix diluted in DMEM/F-12 as required in the datasheet of different batches of hESC-qualified

Matrigel (1 mL per well for coating). The coated plate is kept at room temperature for at least 1 h before using.

2. Use a microscope to visually identify regions of differentiation. Morphologically distinguishable differentiated cells are mechanically removed using a scalpel while viewing under a microscope at 5× magnification.

3. After cutting off differentiated cells, aspirate medium from the well and rinse with DMEM/F-12 (2 mL/well).

4. Add 1 mL per well of Dispase. Place the plate at 37 °C for 7 min (see Note 18).

5. Remove Dispase and gently rinse each well three times with 2 mL of DMEM/F-12 per well.

6. Add 1 mL/well of mTeSR^{TM}1 medium and scrape colonies off with a cell scraper. Ensure that cells are maintained as aggregates.

7. Remove the hESC-qualified Matrigel in the coated 6-well plate. Plate the hESC aggregates with mTeSR^{TM}1 medium onto the new plate (2 mL/well). If the colonies are at an optimal density, the cells can be split every 4–7 days using 1:6–1:10 splits (see Note 19).

8. Move the plate in several quick, short, back-and-forth, and side-to-side motions to disperse cells across the surface of the wells to ensure that newly seeded colonies are evenly dispersed across the entire surface of a coated plate rather than clumped in the middle. Place the plate in a 37 °C incubator.

3.2 Differentiation of hESC in the Neuroepithelial Cyst Model

When hESCs grown in mTeSR^{TM}1 medium are ready to passage, it is also the time to start differentiation experiments.

1. Warm aliquoted Dispase, DMEM/F-12, and N2B27 medium in a 25 °C water bath. Thaw aliquoted BD Matrigel on ice.

   Repeat steps 2–5 in the protocol for passaging the hESCs (see Sect. 3.1 Maintenance of hESCs.). It is critical to remove differentiated cells before Dispase treatment to make sure cyst preparation starts from pure hESCs.

6. Add 1 mL/well of N2B27 medium and scrape colonies off with a cell scraper. Ensure that cells are maintained as aggregates (see Note 20).

7. Transfer the detached cell aggregates (diameter: 50–100 μm) from 1 well of a 6-well plate (~1 × 10^6 cells) to a 15 mL falcon tube.

8. Centrifuge the aggregates at the speed of 700 rpm (92 × g) for 1 min at room temperature (15–25 °C).

9. Aspirate the supernatant. For each well of hESC aggregates collected in the 15 mL falcon tube, add 10–15 μL N2B27 medium. Place the tube on ice.
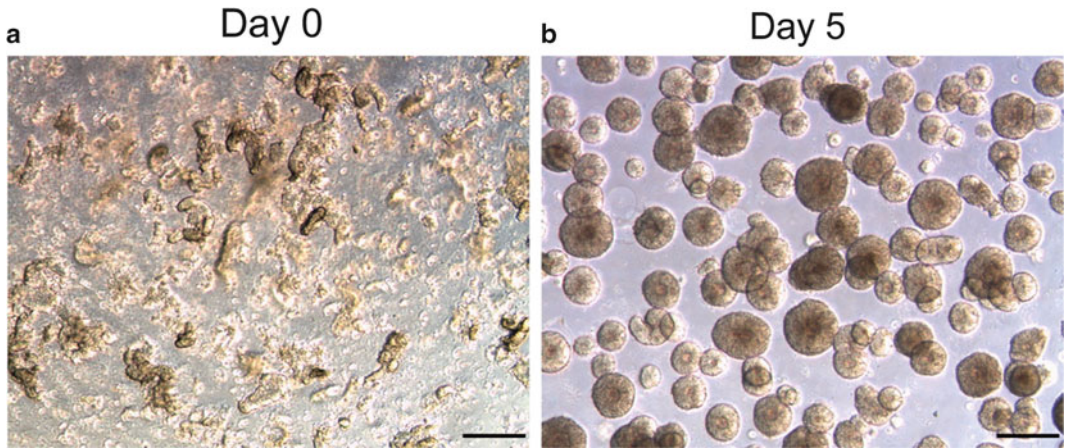
Fig. 1 Efficient generation of neuroepithelial cysts from hESCs in a Matrigel-based 3D neuroepithelial cyst model. (**a**) hESC aggregates were embedded in Matrigel at Day 0. (**b**) hESC aggregates formed neural tube-like cysts with a single lumen in Matrigel by Day 5. Scale bar, 50 μm

10. Add 100–150 μL Matrigel into the 15 ml tube on ice. Resuspend pellet by very gently pipetting up and down with a P200 micropipetter for three times. Cut the pipette tip using the scissors to avoid destroying the cell aggregates during resuspension in Matrigel (see Note 21).

11. Around 50 μL of Matrigel containing the cell aggregates is added onto one glass bottom dish (MatTek Corporation P35G-1.5-14-C). Ensure the Matrigel covers the majority of the glass part of the dish to make a thin layer of Matrigel with cell aggregates in. Avoid a thick Matrigel drop (see Note 22). Place the glass bottom dishes at 37 °C for gelling (8–12 min).

12. Add 2 mL of N2B27 medium in each glass bottom dish after the Matrigel drops gel. The medium is changed every 3–5 days (see Note 23). After 5 days of differentiation in Matrigel, almost all hESC aggregates form neuroepithelial cysts with a single lumen (Fig. 1). The hESC-derived cysts acquire retinal identity (22).

3.3 Differentiation of RPE Cells on Transwell Filters

The hESC-derived cysts at Day 5 (see Note 24) are used for further differentiation toward RPE on transwell inserts.

1. Warm PBS, TrypLE, and N2B27 medium in a 25 °C water bath.

2. Coat the 6.5 mm Transwell® insert with growth factor-reduced-Matrigel. Growth factor-reduced-Matrigel is diluted in DMEM/F-12 (1:20, 100 μL per insert) for coating. The coated plate is kept at room temperature for at least 1 h before using.

3. Aspirate N2B27 medium from the cyst culture and rinse with PBS (2 mL/dish).

4. Prepare a 15 mL falcon tube on ice. Add 5 mL ice-cold Cell Recovery Solution in the tube.

5. Aspirate PBS in one dish and carefully take off the glass coverslip on the bottom of the dish by scalpel (see Note 25). Scrape off the Matrigel drop from the coverslip and put the Matrigel drop containing cysts into the 15 mL falcon tube with ice-cold Cell Recovery Solution in. Collect Matrigel drops from 4 to 5 dishes into one falcon tube.

6. Place the falcon tube on ice for ~30 min. Gently tip the tube up and down from time to time.

7. When there are no visible Matrigel pieces in Cell Recovery Solution and all the cysts are released, keep the tube standing in ice for ~10 min. Large cysts are permitted to sink down to the bottom of the tube by gravity to get rid of the few non-cyst cells. Cysts still remain as intact cysts at this step.

8. Carefully aspirate Cell Recovery Solution from the tube without destroying the soft cyst pellet and add 2 mL PBS.

9. Centrifuge the tube containing the cysts at 300 rpm ($17 \times g$) for 1 min at room temperature (15–25 °C).

10. Aspirate the supernatant. Add 1 mL of diluted TrypLE (dilute TrypLE 1:6 in PBS). Pipet it up and down with a P1000 micropipetter 1–2 times to resuspend the cysts. Place the tube into a 37 °C water bath for 12 min. Very gently flip the tube during the 4-min incubation (see Note 26).

11. After the 12-min incubation in the water bath, add 75 μL Soybean Trypsin Inhibitor solution, pipet the remaining cysts up and down with a P1000 micropipetter several times in the cell culture hood to better dissociate the cysts until there are no visible cell aggregates. Keep the pipetting step shorter than 1 min.

12. Centrifuge the dissociated cysts at 1,000 rpm ($188 \times g$) for 2 min at room temperature (15–25 °C).

13. Aspirate the supernatant. Add 1–2 mL N2B27 medium. Resuspend the cells by pipetting. If the starting amount of cysts is too small, add less medium for resuspension to avoid an additional centrifugation step after counting.

14. Rinse a 40 μm-cell strainer with 1 mL N2B27 medium. Let the resuspended cells go through the cell strainer to get rid of the big aggregates that are not well dissociated. Rinse the cell strainer again with 1 mL N2B27 medium.

15. Count the cells using a hemocytometer.
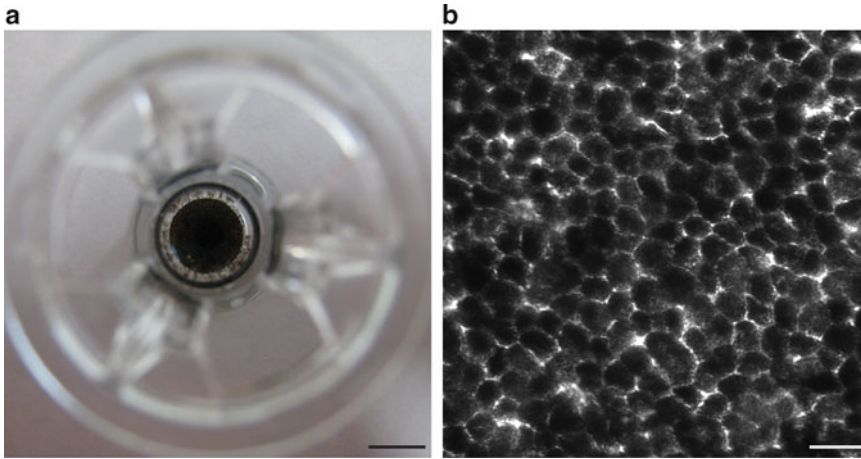
Fig. 2 Directed differentiation of hESC-derived cysts to RPE on transwell filters. (**a**) *Top view* of a transwell insert at 30 days of culture showing the appearance of a pigmented cell sheet. (**b**) A higher magnification of hESC-derived RPE cells at Day 30 displayed *polygonal shape*. Scale bars, 5 mm (**a**), 50 μm (**b**)

16. Aspirate the growth factor-reduced-Matrigel in the Transwell insert and plate 100 μL of the cells onto it at a density of $2\text{--}4 \times 10^5$ cells/well (see Note 27). The lower chamber of the transwell is filled with 600 μL of N2B27 medium.

17. One day after cell seeding (counted as Day 6), attached cells are rinsed twice with RPE medium. The cells are then kept in RPE medium with or without 20–100 ng/mL recombinant Human/Mouse/Rat Activin A (see Note 28). The medium is changed every 2–3 days. The emergence of pigmented cells starts from Day 15 to Day 25. It varies on different cell lines. By Day 30, a confluent pigmented cell sheet has been formed. More than 95 % of the cells in culture get pigmented (Fig. 2).

4   Notes

1. Matrigel should be thawed at 4 °C overnight before aliquoting. Avoid repeated freezing and thawing. The aliquots should be thawed on ice up to twice before use.

2. To make 10 mg/mL Soybean Trypsin Inhibitor solution, dissolve 25 mg soybean trypsin inhibitor in 2.5 mL sterile water. Prepare 100 μL aliquots and store at −20 °C.

3. To make PBS/0.1 % BSA, dissolve 10 μg BSA in 10 mL PBS. Prepare 1 mL aliquots and store at −20 °C.

4. To make 100 mg/mL Transferrin, dissolve 500 mg Transferrin in 5 mL sterile water. Prepare 500 μL aliquots and store at −20 °C.

5. To make 75 mg/mL BSA solution, weigh 375 mg BSA, and make it to 5 mL with PBS. Prepare 350 μL aliquots and store at −20 °C.

6. To make 0.6 mg/mL Progesterone, dissolve 1.2 mg Progesterone in 2 mL ethanol. Prepare 50 μL aliquots and store at −20 °C.

7. To make 160 mg/mL Putrescine, dissolve 160 mg Putrescine in 1 ml sterile water. Prepare 50 μL aliquots and store at −20 °C.

8. To make 3 mM Sodium selenite, weigh 2.59 mg Sodium selenite powder and dissolve it in 5 mL sterile water. Prepare 200 μL aliquots and store at −20 °C.

9. To make 20 mg/mL Insulin, 100 mg Insulin from bovine pancreas is dissolved in 5 mL sterile water. Prepare 630 μL aliquots and store at −20 °C. When preparing N2-supplement, we usually add Insulin as the last component. After adding Insulin, the solution may turn cloudy.

10. Keep the N2-supplement aliquots at −20 °C for up to 3 weeks before use.

11. To make β-mercaptoethanol solution, add 10 μL β-mercaptoethanol into 1.4 mL sterile water. Always work with β-mercaptoethanol inside a chemical fume hood.

12. To make Pyruvate + Glutamine solution, mix 8.25 mL Sodium Pyruvate 100 mM Solution with 5.5 mL L-Glutamine 200 mM. Prepare 500 μL aliquots and store at −20 °C.

13. To make Glutamine + β-mercaptoethanol solution, add 2.8 μL β-mercaptoethanol into 2 mL L-Glutamine 200 mM. Always work with β-mercaptoethanol inside a chemical fume hood.

14. The cells with a passage number less than 50 can be used for differentiation experiments.

15. The hESC colonies should be kept as big as possible during passaging before suspending in mFreSR™ for storage.

16. There is an approximate 24-h window that is optimal for passaging. If colonies are passaged too early or too frequently, the cells may not attach well. If colonies are passaged too late, the culture will begin to show signs of differentiation such as the emergence of cell types with different morphologies. Less than 5 % spontaneous differentiation in culture is considered as high quality.

17. The complete mTeSR™1 medium should be stored at 4 °C and used within 2 weeks.

18. After 7 min of incubation with Dispase, the hESC colony edges will appear slightly folded back but remain attached to the plate.

19. The cell aggregates from 1 well of a 6-well plate can be plated in 6–10 wells of a 6-well plate. If the cell colonies are too dense or too sparse, adjust the split ratio accordingly.

20. We don't pipet the cell aggregates up and down. Try to avoid huge aggregates when scraping off the cell colonies.

21. Cyst formation is high-density dependent. The volume of Matrigel to be used is adjustable.

22. To avoid a thick Matrigel drop in the center of the dish, we slightly slap the dish onto the operation stage of cell culture hood immediately after seeding.

23. When the density of embedded cell aggregates in Matrigel is too high, the N2B27 medium in culture may turn yellowish after 3 days. We then change the medium to fresh N2B27 medium.

24. The day when cell aggregates are embedded in Matrigel is counted as Day 0.

25. Taking the glass coverslip off the dish is just to make the Matrigel drop easily accessible.

26. Try to avoid cysts sticking to the wall of the tube by very gentle flipping.

27. The efficiency of RPE differentiation is density dependent. The best seeding density might differ between different hands based on the cell viability after TrypLE treatment. A titration experiment for optimizing the seeding density (range from 1 to 10×10^5 cells/well) is necessary.

28. The RPE differentiation is Activin A-dependent. In our experiments, H9 cells do require exogenous Activin A for RPE differentiation. But H1 cells do not. It has been proved that Activin-like signaling is indeed required and is presumably made endogenously during RPE differentiation from H1 cells (22).

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Efficient Production of Photoreceptor Precursor Cells from Human Embryonic Stem Cells

Anat Yanai, Christopher Laver, Aaron W. Joe, and Kevin Gregory-Evans

Abstract

Transplantation of photoreceptor precursor cells (PPCs) differentiated from human embryonic stem cells (hESCs) is a promising approach to treat common blinding diseases such as age-related macular degeneration and retinitis pigmentosa. However, existing PPC generation methods are inefficient. To enhance differentiation protocols for rapid and high-yield production of PPCs, we focused on optimizing the handling of the cells by including feeder-independent growth of hESCs, using size-controlled embryoid bodies (EBs), and addition of triiodothyronine (T3) and taurine to the differentiation medium, with subsequent removal of undifferentiated cells via negative cell-selection. Our novel protocol produces higher yields of PPCs than previously reported while reducing the time required for differentiation, which will help understand retinal diseases and facilitate large-scale preclinical trials.

Keywords: Photoreceptor precursor cells, hESC, Feeder-free, Embryoid body (EB), Negative cell selection, Flow cytometry

1 Introduction

Retinal degenerations resulting in irreversible vision loss, such as age-related macular degeneration (AMD) and retinitis pigmentosa (RP), affect millions of people around the world. These and other retinal degenerations lead to loss of cells in the retinal pigment epithelium and/or neurosensory retina, which can ultimately lead to blindness. Although there are some effective therapies for "wet" AMD (e.g., anti-VEGF agents and photodynamic therapy), there are no available treatments for most retinal degenerations including "dry" AMD, RP, and other hereditary retinal dystrophies.

Cell transplantation is a promising approach for replacing damaged, degenerating cells of the neural retina. However, finding the optimal source of transplantable cells and achieving efficient production of sufficient amounts of the desired cells remain challenging. It has been proposed that the appropriate cells for transplantation are ones that have been partially differentiated towards the retinal fate (1) although the optimal stage has yet to be determined.

In the past several years, significant advances have been made in obtaining and generating cells derived from fetal retinal tissue (1),

human adult stem cells (2, 3), human embryonic stem cells (hESCs; (4)), and induced pluripotent stem cells (iPSCs; (5)). Most work has been focused on hESCs as these stem cells have a remarkable capacity for self-renewal and can differentiate into any cell type in the body under the right conditions. These characteristics make them an ideal source for cell replacement therapies.

Exposing hESCs to growth factors and modulators of developmental signaling pathways that mimic normal retinal development has successfully directed undifferentiated hESCs to both retinal pigment epithelium (6, 7) and neural retina cell fate (8, 9). Most of the reported protocols begin with the formation of embryoid bodies (EBs), which are sphere-like structures derived from undifferentiated hESC colonies (10, 11). These EBs were formed by enzymatically detaching hESC colonies and allowing them to "curl" and form a sphere. Using these techniques resulted in a differentiated cell population that consisted of a mixture of most cell types of the neural retina (e.g. ganglion cells, amacrine cells, bipolar cells, horizontal cells, and photoreceptor cells) (6–9).

One of the limiting factors in the production of meaningful amounts of retinal precursor cells for preclinical and clinical studies has been the time-consuming nature of the differentiation protocols (up to 150 days) that often result in low yields of the desired cells (8, 9, 12). As a consequence, the differentiation process can also be very expensive.

Our goal was to develop a method to efficiently produce large amounts of retinal precursor cells. We focused on developing a protocol for just one cell type, photoreceptor precursor cells (PPCs), rather than the production of a range of retinal cells because that is the cell type most in demand for preclinical trials. We modified information from several previous studies and experimented with EBs of variable sizes.

The resulting novel protocol begins with the feeder-free growth and the use of chemically defined media for hESC maintenance, reducing the variability associated with growth on feeder cells. The second stage is the use of size-selected EBs with a diameter of ~200 μm, containing 1,000 cells, to synchronize the differentiation process and finally, the addition of taurine and T3 during the differentiation period. This method produces, in 17 days, a cell population enriched for PPCs that express BLIMP1 (a protein expressed in early photoreceptor development) (13, 14), CRX, recoverin, and s-opsin.

The protocol outlined in this chapter describes in detail the maintenance and expansion of undifferentiated hESCs grown in feeder-free systems and the multistep generation of PPCs over the course of 17 days. The protocol then summarizes a method we use to confirm the identity of the PPCs: RT-qPCR and flow cytometry. We also outline the procedure to further enrich PPC purity using magnetic separation to remove undifferentiated cells still expressing the hESC marker SSEA-4.

2   Materials

2.1   Culture of Undifferentiated hESCs

1. The hESC line used: WA-09 (WiCell Research Institute).
2. hESC growth media: Complete TeSR™2 (STEMCELL Technologies).
3. Dispase (1 mg/ml in DMEM/F-12; STEMCELL Technologies).
4. Serological glass pipettes.

2.2   Surface Coating for Adherent Cell Growth of hESCs and Differentiating PPCs

1. Growth Factor Reduced Matrigel™ (BD Biosciences).
2. DMEM/F-12 (Life Technologies).
3. Six-well plates with Nunclon-treated surface (Nunc).

2.3   Generation of PPCs from Undifferentiated hESCs

1. Accutase™ (STEMCELL Technologies).
2. Dispase.
3. AggreWell™ 400 plate (STEMCELL Technologies).
4. Cell strainers (40 and 100 μm pores; Fisher or STEMCELL Technologies).
5. Ultra-Low Attachment plates (Corning).
6. EB formation medium: TeSR™2 supplemented with 10 μM Rho-Associated Coil Kinase (ROCK) inhibitor (STEMCELL Technologies).
7. EB resuspension medium: DMEM/F12, 10 % knockout serum replacement, custom B-27 and N-2 supplements (Life Technologies), 1 ng/ml mouse noggin, 1 ng/ml recombinant human Dkk-1 and 5 ng/ml recombinant human IGF-1 (R&D Systems).
8. PPC differentiation medium: DMEM/F12, custom B-27 and N-2 supplements, 10 ng/ml mouse noggin, 10 ng/ml recombinant human Dkk-1, 10 ng/ml recombinant human IGF-1 and 5 ng/ml recombinant human basic FGF (Life Technologies). When indicated, 20 mM taurine (Sigma) and 40 ng/ml triiodothyronine (T3; Sigma) are added to the differentiation medium as specified below.
9. The factors: Noggin, Dkk-1 and basic FGF are dissolved in Dulbecco's PBS (DPBS; Life Technologies) containing 0.1 % bovine serum albumin (BSA) at a concentration of 10, 100 or 2 μg/ml, respectively. IGF-1 was dissolved in DPBS at a concentration of 200 μg/ml. All factors should be stored in single-use aliquots at −80 °C.
10. T3 is first dissolved in 1 N NaOH at 100 mg/ml and then diluted in DMEM to 40 μg/ml. Stored in single-use aliquots at −80 °C. Taurine powder can be directly dissolved in PPC differentiation medium and filter-sterilized before use.

2.4 Magnetic Removal of Undifferentiated Cells	1. SSEA-4 Positive Selection kit (STEMCELL Technologies).
	2. EasySep® Magnet (STEMCELL Technologies).
	3. Accutase.
	4. Polystyene 5 ml round-bottom tubes (Falcon).
	5. Selection buffer: DPBS, 2 % Fetal Bovine Serum (FBS) and 1 mM EDTA.

2.5   Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

1. Aurum™ Total RNA Fatty and Fibrous Tissue kit (Bio-Rad).
2. iScript™ cDNA Synthesis kit (Bio-Rad).
3. UltraPure™ DNase/RNase-free Distilled water (Life Technologies).
4. FAM- or VIC-labeled TaqMan® Gene Expression primers (Life Technologies).
5. TaqMan® Universal Master Mix (Life Technologies).
6. MicroAmp® 96-well reaction plate and the corresponding Optical Adhesive Covers (Applied Biosystems).
7. Real Time PCR System, such as the ViiA™ 7 (Applied Biosystems).

2.6   Flow Cytometry

2.6.1   Cell Surface Labeling

1. Accutase.
2. Blocking buffer: DPBS with 10 % human Serum (Sigma).
3. Washing and resuspension buffer: DPBS with 2 % FBS.
4. Polystyene 5 ml round-bottom tubes.
5. Primary antibody, as desired (*see* **note 1**).
6. Alexa Fluorophore-conjugated secondary antibody.
7. Propidium iodide (Life Technologies).

2.6.2   Intracellular Labeling

1. Accutase.
2. DPBS with 2 % FBS.
3. DPBS with 2 % paraformaldehyde (PFA).
4. Saponin Permeabilization Buffer (SPB): DPBS, 0.1 % Bovine Serum Albumin (BSA) and 0.2 % saponin (Sigma).
5. Polystyene 5 ml round-bottom tubes.
6. Primary antibody, as desired (*see* **note 2**).
7. Alexa Fluorophore-conjugated secondary antibody.

3   Methods

3.1   Coating Plates with Matrigel™

Both human embryonic stem cells (hESCs) and differentiating PPCs grow on tissue culture plates coated with Matrigel. Matrigel is supplied as a frozen solution. Thaw on ice overnight in a 4 °C

refrigerator and make single-use aliquots into precooled 1.5 ml centrifuge tubes using precooled pipette tips (*see* **note 3**). It is important to keep all the solutions cold prior to coating, as Matrigel will solidify rapidly at room temperature.

1. For coating a 6-well plate, remove an aliquot of 0.5 mg Matrigel from the −80 °C freezer and place on ice (*see* **note 3**).

2. Dispense 5 ml of cold DMEM/F-12 into a 15 ml conical tube.

3. Add 1 ml cold DMEM/F-12 to the frozen Matrigel aliquot and pipette up and down to dissolve. Quickly transfer the Matrigel solution to the conical tube and mix well.

4. Distribute 1 ml of diluted Matrigel per well, swirl the plate to ensure uniform coating and incubate at room temperature for at least 1 h.

5. If the coated plate is to be used more than 1 h later, add 1 ml of DMEM/F-12 to prevent the surface from drying (*see* **note 4**).

3.2 Culture of Undifferentiated Human Embryonic Stem Cells

1. Culture the hESC line WA09 in TeSR™2 growth medium in a 37 °C incubator in 5 % CO_2. Change medium daily and manually remove spontaneously differentiating cells, as needed.

2. Passage cells every 4–6 days by incubating the cells with 1 ml Dispase per well. When colonies start to "curl" and detach from the surface (typically 5–10 min), remove the Dispase solution and carefully wash colonies two times with DMEM/F12. Using a glass pipette and 2 ml of fresh DMEM/F-12, the colonies should be gently scraped off the surface and collected in a 15 ml conical tube. Use 2 ml fresh DMEM/F-12 to collect the remaining colonies and add to the conical tube. Centrifuge the cell suspension for 5 min at 300 × *g* and resuspend the cell pellet in fresh TeSR™2 by slowly pipetting up and down to break the colonies to smaller pieces. Distribute the colonies on Matrigel coated plates, according to the split ratio required, and slowly move the plates side to side and front to back to uniformly disperse the colonies.

3.3 Generation of PPCs from hESCs

The differentiation of hESCs to PPCs is a multistep procedure (Fig. 1). The first step is the generation of size-controlled embryoid bodies (EBs), which we have recently shown can be used to enhance the yield of PPCs (15). The generation of size-controlled EBs can be performed in two ways; manual selection of medium-sized EBs, which result in the highest proportion of PPCs when separated from a mixed-size EB population, or by using the commercially available AggreWell 400 plate to generate 1,000-cell EBs (*see* **note 5**). The protocol below outlines both methods. Representative images of the EBs generated by this protocol can be seen in Fig. 2.
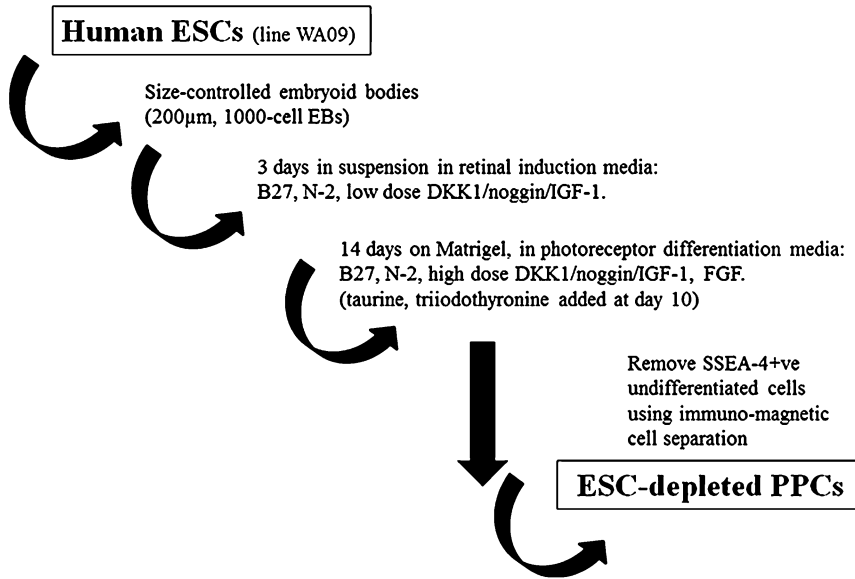
Fig. 1 Schematic representation of the photoreceptor precursor cells differentiation protocol
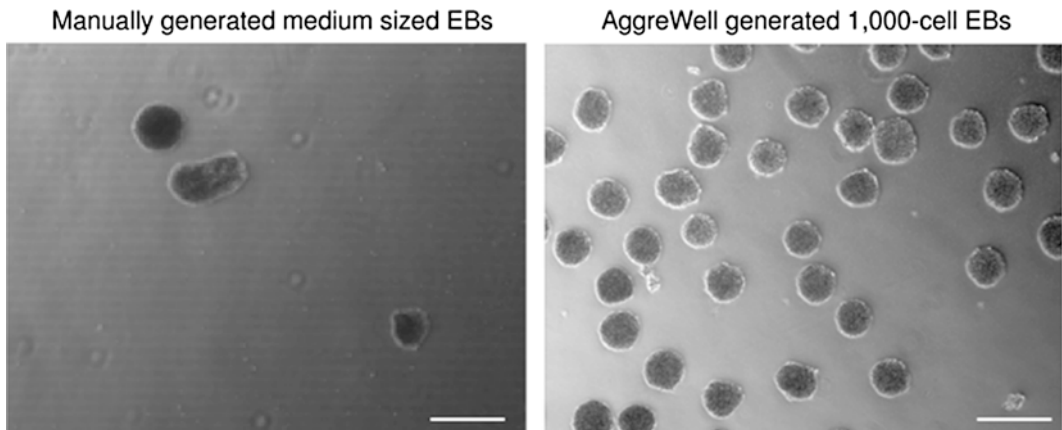
Fig. 2 Representative images of the EB sizes produced in this protocol. Scale bar is 400 μm

3.3.1 Generation and Manual Selection of Medium-Size EBs

1. Incubate hESCs with Dispase at 37 °C until colonies begin to peel off the plate (typically 20–30 min). Gently wash the colonies with the Dispase solution, transfer to a 15 ml conical tube, and collect residual colonies with 2 ml of DMEM/F-12 per well. Let the colonies settle to the bottom of the tube for ~5 min, aspirate the supernatant, and wash the colonies with 4–6 ml of fresh DMEM/F-12. After colonies have settled down and the supernatant aspirated the second time, resuspend the colonies in EB resuspension buffer and place on Ultra-Low

Attachment plates in a volume of 5 ml per well for 3 days in the 37 °C incubator (*see* **note 6**). Move the plates slowly from side to side and front to back 3–4 times daily, to prevent sticking of EBs.

2. At day 4 of incubation, place the plate in the sterile tissue culture hood and manually remove the largest EBs (diameter of ~400 μm or more) using a 200 μl pipette tip, and pass the remaining suspension over 100 μm nylon cell strainer (*see* **note 7**). Single cells and small EBs will flow through. Turn the cell strainer so that the EBs are facing a new 50 ml conical tube and elute the "trapped" EBs in 2–4 ml PPC differentiation medium. Distribute the eluted EBs on Matrigel-coated plates and move the plate from side to side and front to back to evenly distribute the EBs (*see* **note 8**).

3.3.2 Manufacture of 1,000-Cell EBs Using AggreWell Plates

1. Wash one or more wells of hESCs once with DMEM/F-12, aspirate and replace with 0.75 ml of room temperature Accutase per well. Incubate the cells at 37 ° C for 5–10 min, until most cells have dislodged. With a 1 ml tip, pipette the cells several times to dissociate any remaining clumps and collect the cell suspension of all wells into a conical tube. Collect the remaining cells with 4 ml DMEM/F-12 per well into the same tube and pellet cells by centrifugation for 5 min at $300 \times g$. Resuspend the cell pellet in 1 ml EB formation media and count the viable cells with trypan blue. To generate 1,200 EBs with 1,000 cells per EB, add ~1.2×10^6 cells to one well of the AggreWell400 and centrifuge the AggreWell plate for 3 min at $100 \times g$. Place the AggreWell plate in a 37 ° C incubator for 24 h.

2. Harvest EBs by pipetting the medium in the well up and down several times with a 1 ml tip to dislodge most of the EBs. Pass the EB suspension through a 40 μm cell strainer and wash the AggreWell five more times with 1 ml of DMEM/F-12 to dislodge the remaining EBs. Pass all the suspensions through the same cell strainer.

3. Invert the cell strainer so that the EBs are facing a 50 ml conical tube. Release the EBs from the cell strainer in 5 ml of EB resuspension buffer and distribute evenly to two or more wells (*see* **note 9**). The final volume in each well should be 5 ml of EB resuspension buffer.

4. Incubate the EBs for 3 days in the 37 °C incubator. To prevent sticking of EBs, move the plate slowly from side to side and front to back 3–4 times daily. At day 4 of incubation, collect all the EBs in a 50 ml conical tube and centrifuge for 5 min at $100 \times g$. Aspirate the medium and carefully resuspend the EBs in PPC differentiation medium (2 ml per well, *see* **note 10**).

Evenly distribute the EBs on Matrigel coated plates by moving the plate from side to side and front to back.

3.3.3 Adherent Growth of PPCs

1. Culture the cells, originating from EBs generated by both manual size selection and AggreWell plates, in PPC differentiation medium for 2 weeks without passaging. Change the culture medium every other day. On the 7th day of adherent growth, supplement the PPC differentiation medium with taurine and T3 (20 mM and 40 ng/ml, respectively) and continue with the same feeding schedule.

2. After 2 weeks of adherent growth, cells may be frozen in PPC differentiation medium containing 10 % DMSO for future use, or they may be harvested for analysis of differentiation stage.

3.4 Analysis of Differentiation Stage Using RT-qPCR

Relative gene expression can be quantified with the TaqMan® primer/probe system. Use FAM-labeled primers for the gene of interest and VIC-labeled primers for an internal control (such as GAPDH). All the details described below refer to the use of the TaqMan® system and the ViiA™ 7 Real Time PCR system.

1. Following 17 days of differentiation, isolate total RNA from cell cultures using the Aurum™ Total RNA Fatty and Fibrous Tissue kit according to the manufacturer's instructions.

2. For each sample to be processed, use the iScript™ cDNA Synthesis kit to reverse transcribe 1 μg of total RNA according to the manufacturer's instructions.

3. Dilute the resulting cDNA 1:10 by adding 180 μl UltraPure™ distilled water. Further dilute a portion of the 10x diluted sample 1:2 to make a "working stock" of $20\times$ (*see* **note 11**). All samples should be stored at -20 °C.

4. The RT-qPCR reaction mix is as follows (scale up according to the number of samples required):

   6 μl UltraPure™ distilled water

   10 μl TaqMan® Universal Master Mix

   1 μl FAM-labeled TaqMan® Gene Expression assay (gene of interest; *see* **note 12**)

   1 μl VIC-labeled TaqMan® Gene Expression assay (internal control)

   2 μl cDNA

   20 μl total volume per reaction

5. Load all samples into the 96-well plate, seal the plate with an optical adhesive film, centrifuge the plate briefly and load the plate into the instrument. Thermal cycling conditions should be as follows: initial incubation consisting of 2 min at 50 °C and

then 10 min at 95 °C, followed by 40 cycles of: 15 s at 95 °C and 1 min at 60 °C.

6. Analyze the data using the comparative C_T method to calculate the fold change in the expression of the gene of interest compared to a calibrator (such as undifferentiated cells).

3.5   Flow Cytometry   The protocols detailed below have been optimized for the antibodies we used to detect specific cell surface and intracellular antigens (SSEA-4 and CRX, respectively; *see* **notes 1** and **2**).

3.5.1   Cell Surface Labeling

1. To collect and make a single cell suspension, use Accutase as described in Sect. 3.3.2. Note that differentiated cells detach quicker with Accutase than undifferentiated hESCs (less than 5 min). Use ice-cold DPBS to resuspend the resulting cell pellet.

2. Count the cells and distribute ~2×10^5 cells per sample into a 1.5 ml conical tube.

3. Centrifuge the tubes at $300 \times g$ for 5 min and resuspend the cell pellet in 100 μl DPBS/10 % human serum. Block on ice for ~10 min.

4. Add 1 μl of anti-SSEA-4 antibody directly to each sample, vortex briefly, and incubate on ice for 30 min.

5. Add 1 ml of DPBS/2%FBS, vortex briefly, and centrifuge the tubes at $300 \times g$ for 5 min.

6. Aspirate the supernatant and resuspend each cell pellet in 100 μl of Alexa-488 goat anti-mouse IgG diluted 1:400 in DPBS/2 % FBS.

7. Vortex briefly and incubate on ice in the dark for 15 min.

8. Aspirate the supernatant, add 1 ml of DPBS/2%FBS, vortex briefly, and centrifuge the tubes at $300 \times g$ for 5 min.

9. Aspirate the supernatant and resuspend the cell pellets in 300 μl of DPBS/2%FBS supplemented with propidium iodide (diluted 1:1,000). Transfer the cell suspension into a 5 ml round-bottom tube.

10. Analyze on a flow cytometer instrument, such as the BD Biosciences FACSCalibur.

3.5.2   Intracellular Labeling

1. Prepare a single-cell suspension as described above, but resuspend the cell pellet in ice-cold DPBS/2 % FBS.

2. Count the cells and distribute ~8×10^5 cells per sample into a 1.5 ml conical tube.

3. Centrifuge the tubes at $300 \times g$ for 5 min and fix the cells by resuspending the pellet in 250 μl DPBS/2 % PFA and incubating for 15 min on ice.

4. Add 1 ml of DPBS/2%FBS, mix carefully, and centrifuge the tubes at 300 × *g* for 5 min.

5. Aspirate the supernatant and permeabilize the cells by resuspending the pellet with 0.5 ml Saponin Permeabilization Buffer (SPB) and then incubate for 15 min at room temperature.

6. Centrifuge the tubes at 300 × *g* for 5 min, aspirate the supernatant and resuspend each cell pellet in 100 μl CRX antibody diluted 1:200 in SPB.

7. Incubate at room temperature for 30 min.

8. Perform two washes by the addition of 1 ml of SPB to each sample, mix carefully and centrifuge at 300 × *g* for 5 min.

9. After aspirating the supernatant for the second time, resuspend each cell pellet in 100 μl of Alexa-488 goat anti-rabbit IgG diluted 1:400 in SPB.

10. Incubate on ice for 20 min and perform two washes as described above.

11. Resuspend cell pellets in 300 μl of DPBS/2 % FBS and transfer the cell suspension into a 5 ml round-bottom tube.

12. Analyze on a flow cytometer instrument, such as the BD Biosciences FACSCalibur.

3.6 Magnetic Removal of Residual Undifferentiated Cells Using EasySep® Magnetic Selection System

In order to reduce the risk of tumors resulting from residual undifferentiated cells, cells expressing hESC surface markers, such as stage-specific embryonic antigen-4 (SSEA-4), can be magnetically removed from the total cell population to enrich for the desired differentiated cells. The procedure below describes the depletion of SSEA-4 expressing cells using the EasySep® positive selection kit.

1. Following 17 days of differentiation collect the total cell population with Accutase, as described above (Sect. 3.3.2) and resuspend in 100 μl of selection buffer (*see* note 13). Note that differentiated cells detach quicker with Accutase than undifferentiated hESCs (less than 5 min).

2. Add 10 μl Selection Cocktail, mix well and incubate at room temperature for 15 min.

3. Mix Magnetic nanoparticles by pipetting up and down several times and add 5 μl to the cell suspension. Mix well and incubate at room temperature for 15 min.

4. Top up the cell suspension with selection buffer to a volume of 2.5 ml. Mix well, place the tube into the EasySep magnet, and set aside for 10 min.

5. Pick up the magnet and invert the magnet and tube, pouring the desired fraction into a new 5 ml tube.

6. Remove the tube with the unwanted cells and place the tube containing the desired fraction inside the magnet for a second round of magnetic separation. Repeat step 5.

7. The enriched cells can now be used for further experiments.

4 Notes

1. For cell surface labeling, we used the anti-human SSEA-4 monoclonal antibody, clone MC-817-70 (gift from Dr. Connie Eaves), which is now commercially available from several companies (e.g. STEMCELL Technologies and Millipore). We used this antibody at a 1:100 dilution.

2. For intracellular labeling, we used the anti-CRX (cone rod homeobox) rabbit polyclonal antibody developed in our lab (16). When using the above protocol it is crucial to titrate the relevant antibody on a sample that is known to express the protein of interest.

3. As mentioned above, Matrigel solidifies rapidly at room temperature. When preparing single-use aliquots for future use, make sure the tubes and tips are precooled in a −80 °C freezer a day before. One 6-well culture dish requires 0.5 mg Matrigel. It is useful to make aliquots of 0.5, 1, and 2 mg Matrigel to coat 1, 2, and 4 plates, respectively.

4. Coated plates can be stored at 4 °C for up to 1 week but need to be warmed to room temperature for at least 30 min before use.

5. We saw a significant improvement in the yield of PPCs when using the Aggrewell™400 to produce 1,000-cell EBs. However, if AggreWell plates are not available, medium sized EBs can be separated from a mixed size EB population to yield the highest percentage of PPCs in comparison to the mixed-size EB population and the small- and large-sized EBs. Refer to our 2013 paper (15) regarding size-controlled EBs.

6. EBs generated from one confluent well of undifferentiated hESCs are transferred into one well of an Ultra-Low Attachment plate. This can be scaled up in the same ratio to generate larger amounts of PPCs.

7. The largest EBs can be distinguished visually. Using a pipette with a 200 μl pipette tip, these EBs can be picked up and discarded.

8. Typically, medium size EBs from several wells of mixed-size EBs were pooled together and evenly distributed onto Matrigel coated plates in a ratio of 1:1.5–1:2 (for example, from two wells of mixed-size EBs in suspension, three wells of medium size EBs were cultured).

9. STEMCELL Technologies recommends incubating <1,000 EBs, generated in AggreWell plates, in one well of low attachment plate. When we generate EBs in one AggreWell we transfer the EBs into two low attachment wells, but when generating 2–3 AggreWells the resulting EBs are incubated in 3–4 low attachment wells, respectively.

10. The plating density of AggreWell EBs should be 500–600 EBs per one well of a 6-well plate, assuming 1,200 EBs are harvested from each AggreWell used.

11. cDNA was typically divided to a "working" sample ($20\times$) and a "back up" sample ($10\times$), to be used later on or in the case of an accidental contamination. The "working" stock is at a concentration of approximately 2.5 ng/µl and we use 2 µl (5 ng) for each RT-qPCR reaction.

12. Each RT-qPCR reaction contains FAM-labeled primer for the gene of interest and a VIC-labeled primer to amplify GAPDH. This way we can normalize all reactions to one internal control in the same reaction.

13. The manufacturer recommends using 100 µl selection buffer for samples containing up to 1.25×10^7 cells. In our laboratory we have not used samples that required a higher volume of selection buffer. However, this volume can be scaled up as required.

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Generation of Megakaryocytes and Platelets from Human Pluripotent Stem Cells

Marjorie Pick

Abstract

Human pluripotent stem cells (hPSC) have the potential to produce any tissue type in the body and thus represent a source of cells for regenerative medicine. Here we have shown that human platelets can be produced from embryonic or induced pluripotent stem cells in a defined culture system. We describe a serum- and feeder-free culture system that enabled the generation of megakaryocyte (Mk) progenitors and functional platelets from hPSCs. After 13 days the differentiated population included precursor cells that formed colonies containing differentiated Mks, and after 20 days these Mks were able to fragment into platelet-like particles that were functional. This protocol represents an important step towards the generation of human platelets for therapeutic use.

Keywords: Embryonic stem cells, Megakaryocytes, Platelets, Feeder-free, Differentiation, Hematopoiesis

1 Introduction

Pancytopenia and thrombocytopenia remain significant clinical problems for patients suffering from a range of medical conditions. Therefore, generating platelets from human pluripotent stem cells could prove to be a useful alternative source to single donor platelets obtained from healthy individuals for transfusion. Defined conditions are critical for the reproducibility of protocols and the removal of materials containing animal products is highly desirable. We have established an animal product-free and stromal cell-free differentiation protocol for differentiating cells towards CD41-positive Mks that then releases platelet-like particles that display functionality. Our laboratory has established protocols based on the generation of homogenous "spin embryoid bodies (EBs)" from hPSCs (1) in a defined medium that is supplemented with cytokines to bias differentiation towards desired outcomes (2, 3). This protocol allowed for the robust generation of CD41$^+$ and/or CD34$^+$ hematopoietic cells that contain Mks progenitors. Furthermore, quantitative PCR

analyses showed that the CD41$^+$ cells express high levels of Mk-associated genes and that the cells display polyploidy characteristic of developing Mks. In addition, the supernatant of day 20 cultures yielded CD41$^+$ CD42b$^+$ platelet-like particles that could be activated by ADP and thus upregulate CD62P.

2   Materials

2.1   Mouse Embryonic Fibroblast (MEF) Medium (notes 1 and 2)

500 ml DMEM medium high glucose (4.5 g/l) (Invitrogen) supplemented with 5 ml Penicillin–Streptomycin (Pen-Strep) Solution (Biological industries) and 5 ml L-Glutamine (Biological Industries) and 10 % fetal bovine serum (Invitrogen).

2.2   Human Pluripotent Stem Cell (hPSC) Medium (notes 1 and 2)

500 ml DMEM/Hams F12 medium (Invitrogen) supplemented with 15 % knockout serum replacer (Invitrogen), 5 ml Non-essential amino acids (Invitrogen) 5 ml Pen-Strep Solution, 5 ml L-Glutamine, 50 mM 2-β Mercaptoethanol (Sigma) and 4 ng/ml recombinant human fibroblast growth factor (rhFGF2) (Peprotech).

2.3   Chemically Defined Medium (notes 1 and 2)

1. IMDM (Invitrogen) and F12 Medium (Invitrogen) are added at a 1:1 ratio—212.4 ml each.
2. 25 ml (5 mg/ml) of recombinant human serum albumin (Novozymes).
3. 5 ml (100×) Lipids (Invitrogen).
4. 5 ml (100×) insulin, transferrin, selenium solution (Invitrogen).
5. 50 mM mono-thioglycerol (Sigma).
6. 25 ml protein free hybridoma medium (Invitrogen).
7. 2 mM glutamax (Invitrogen).
8. 2.5 ml Pen-Strep.

3   Methods

3.1   Preparation of Mouse Embryonic Fibroblasts (MEF) Feeder Layer (note 3)

1. Cells are isolated from 12.5 to 13.5 day mouse embryos into single cells suspension.
2. Cells are grown in 14 mm diameter tissue culture plates (Nunc) until confluent with MEF medium.
3. Once confluent (2–4 days), cells were trypsinized (Trypsin EDTA Solution A, Biological Industries) and split 1–3. This is P1.
4. Once confluent the cells were passaged again with trypsin and split 1–3. This is P2.

5. Once P2 is confluent cells were treated for 2 h with 1 mg/ml of Mitomycin C (Fermentek) to stop cell division.

6. Cells are then trypsinized and counted.

3.2 Human Embryonic or Induced Pluripotent Stem Cells (hPSC) Maintenance (note 4)

1. MEFs were thawed and placed on gelatin (0.1 %, Sigma)-coated tissue culture plates at $2.0–6.0 \times 10^4$ cells/cm^2 and incubated for at least 4 h before adding hPSC cells.

2. Human pluripotent stem cells were maintained in pluripotent state on mitomycin-treated MEF feeder cells in hPSC medium.

3. When confluent, every 3–4 days, cells were enzymatic passaged with trypsin solution A (Invitrogen) as previously described (2, 4).

3.3 Differentiation of hPSCs

1. hPSCs were harvested with TrypLE Select (Invitrogen).

2. Single cells were resuspended in serum-free CDM medium (5) supplemented with 5–15 ng/ml rh Bone Morphogenic Protein (BMP4) (Peprotech), 10–15 ng/ml rh Vascular Endothelial Growth Factor (VEGF) (Peprotech), 10 ng/ml rhFGF2 (Peprotech) and 25 ng/ml rh Stem Cell Factor (SCF) (Peprotech) as described previously (2) (note 5).

3. One hundred microlitres of cell suspension containing 5,000 hPSCs were placed into a well of round-bottom low-adherent 96-well plates (Nunc).

4. Within 24 h, single embryoid bodies (EBs) formed in each well.

5. At 10 days, 72 EBs were transferred to each well of a 6-well flat-bottom tissue culture plate (Nunc) containing 7.2 ml of CDM supplemented with 20–30 ng/ml rh thrombopoietin (TPO) (Peprotech), 25 ng/ml rhSCF and 25–50 ng/ml rh interleukin (IL)-3 (Peprotech).

6. EBs were further cultured for a total of 20 days.

7. At any point after 13 days of differentiation the cells generated in differentiation cultures can be removed and with the use of trypLE select dissociated into a single cell suspension for immunophenotyping, colony assays, or ploidy assessment.

8. At any point after 13 days of differentiation the EBs generated in differentiation cultures can be removed and RB buffer directly added to the pellet for RNA extraction.

3.4 Flow Cytometric Analysis of Differentiated hPSCs (note 6)

1. After 13 days of differentiation cultures were assessed for the presence of hematopoietic cells and megakaryocytes using monoclonal antibodies such as anti-CD34 (BD Biosciences), anti-CD41 (BD Biosciences) and CD45-PE (BD Biosciences).

2. Incubation of the antibodies with cells should be for a minimum of 20 min at 40 °C.

3. Cells were washed in phosphate buffered saline (PBS) to remove unbound antibody.

4. Centrifugation of cells was at $480 \times g$ for 5 min at 40 °C.

5. Before acquisition on the flow cytometer differentiated cells pellets were resuspended in 200 μl of PBS containing propidium iodide (PI) to exclude dead cells.

6. Analysis of differentiation cells was performed on a FACSCalibur using CellQuest Pro software (BD Biosciences).

3.5 Methylcellulose-Based Myeloid Colony Forming Assay

1. Triplicate assays were completed in 24-well tissue culture treated plates (Nunc) with 10,000 cells added per well in 0.5 ml of Methocult™ (Stem Cell Technologies, Canada).

2. Methocult was supplemented with 20 ng/ml rh granulocyte-macrophage-colony stimulating factor (GM-CSF), 50 ng/ml rh SCF, 20 ng/ml rh IL-3, 3 U/ml rh erythropoietin (EPO) and 20 ng/ml rh IL-6 (all from Peprotech).

3. Cells were incubated at 37 °C in 5 % CO_2 for 14 days.

4. Colonies were scored according to their morphology either as GEMM-, GM-, G- or M-CFU.

5. To determine presence of Mks in GEMM-CFUs, colonies were picked from the methylcellulose, dried on a slide and stained with May-Grunwald/Giemsa to detect typical Mk morphology (Fig. 1).

3.6 Collagen-Based Megakaryocyte Colony Forming Assay

1. Duplicate assays were performed in 24-well tissue culture treated plates into which 10,000 cells were mixed with a collagen-based medium (MegaCultTM, Stem Cell Technologies).

2. Megaocult was supplemented with 50 ng/ml rhTPO, 25 ng/ml rhSCF and 10 ng/ml rh IL-3 to stimulate colony formation.

3. After 14 days plates were dried and stained with anti-CD41 antibody and an alkaline phosphatase colorimetric assay according to the kits instructions (Stem Cell Technologies) (Fig. 1a).

3.7 Quantitative Real-Time PCR

1. RNA was extracted from hPSCs at various time points using an RNA extraction kit (GeneAid).

2. Superscript III (Invitrogen) was used to reverse transcribed RNA to cDNA with random hexamer primers.

3. Real-time PCR was performed using Taqman gene expression probes (Applied Bioscience) and the one-step PCR system absolute thermal cycler and software (Applied Bioscience).

4. The comparative cycle threshold (CT) method was used to analyze data, with gene expression levels compared to GAPDH expression as previously described (2) and ddCT used for quantitation (Fig. 2).
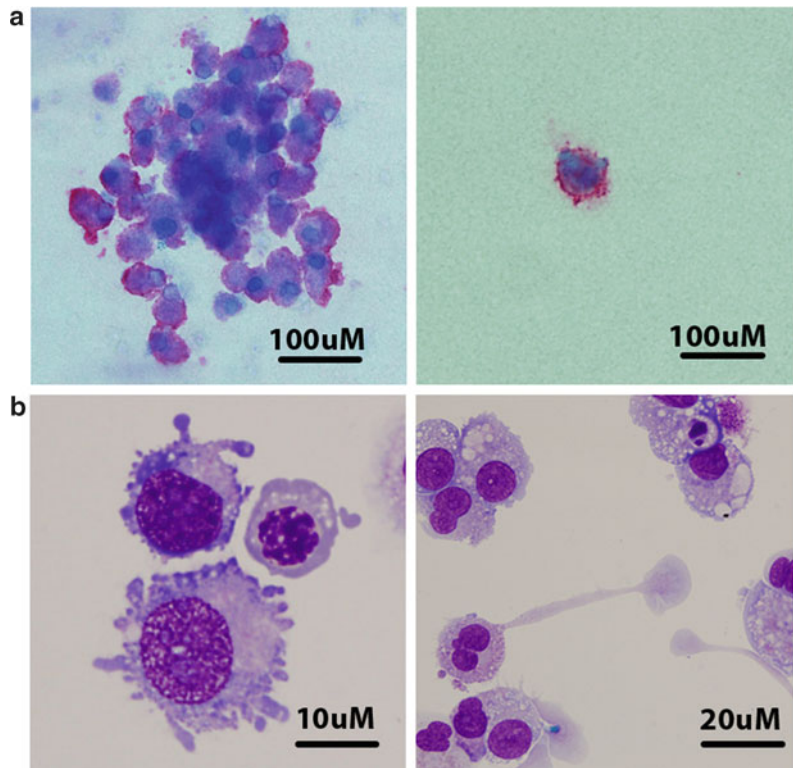
Fig. 1 (**a**) Images of megakaryocytic colonies expressing CD41 cultured from differentiated human ES cells. (**b**) Images of megakaryocytes stained with May-Grunwald/Giemsa picked from colonies generated from hPSCs differentiation cultures
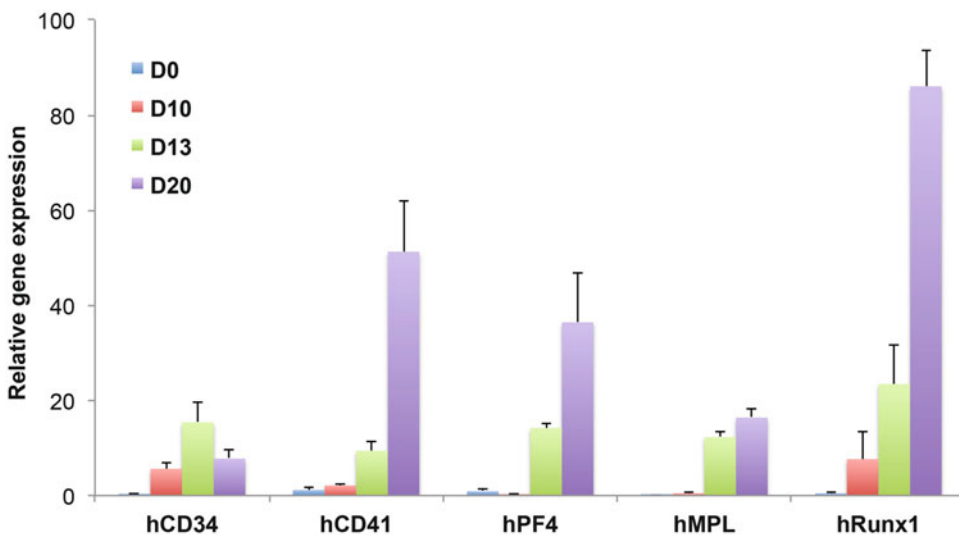
Fig. 2 Hematopoietic gene expression in differentiated hPSCs. cDNA was generated from EBs. Gene expression was quantified by real-time PCR analysis for the indicated genes. Histograms show relative gene expression expressed as mean ± sSEM of five individual experiments

3.8 Analysis of Cell Ploidy (note 7)

1. Megakaryocytes have a distinct characteristic that allows the DNA to double without the cell dividing—polyploidization.
2. This polyploidization or cell ploidy can be analyzed by staining cellular DNA with PI.
3. Single cell suspensions of cells were fixed in cold ethanol at 40 °C overnight.
4. Cells were centrifuged at $850 \times g$ for 5 min.
5. To the resuspended pellet PBS is added.
6. RNase A (2 mg/ml) (Sigma) was added to the resuspended cells and incubated for 30 min at 370 °C.
7. 15 min before cells are ready to be acquired in the flow cytometer, PI (Sigma) was added.
8. At least 50,000 events were collected to allow enough cells in the >8 N peaks to be seen.

3.9 Flow Cytometric Analysis of Platelet-Like Particles (note 8)

1. To harvest platelet-like particles from hPSCs cultures, the supernatants were collected.
2. Centrifugation at $480 \times g$ for 5 min at 4 °C was performed to collect the particles.
3. Immunophenotyping of the particles were performed using monoclonal antibodies. CD41a-FITC and CD42b-PE and stained similarly to previous protocol.
4. Acquisition of the flow cytometry data used log amplification of forward and side scatter to include the small platelet-like particles.
5. Isotype controls for the antibody stains must be included and depends on the supplier of the antibodies.
6. At least 15,000 events were acquired.

3.10 Platelet Activation

1. Platelet-like particles were harvested as described above, pelleted by centrifugation and resuspended in PBS.
2. The pelleted particles were incubated in the presence or absence of 20nM ADP at room temperature form 20 min and then stained for the expression of CD41a-FITC and CD62P-PE (6).
3. Cord blood platelets were included as a positive control (6) (note 8).
4. Isotype controls for the antibody stains are essential.
5. 15,000 events were acquired on the flow cytometer.
6. Platelet-like particles that were activated by ADP will express higher levels of the CD62P marker and can be seen as an increase in intensity or shift in the histogram (3, 6).

4   Notes

1. All components of the medium should be mixed together and filtered with an 0.2 μm size media filter (Nunc) to improve sterility.

2. Complete medium should be stored at 4 °C for up to 1 month.

3. To store large numbers of MEFs for long term $1–2 \times 10^6$ of them can be placed into a cryopreservation tube (Nunc) together with MEF medium and 10 % DMSO and aliquots of MEFs were kept in liquid nitrogen for storage until use. The MEFs can be then thawed and used according to need.

4. HPSCs should be checked for chormosomal anbnormalitites via karyotyping every 6 months and stemness of hPSCs must be checked with the use of antibody expression of Tra-1—60 and Oct4 every 12 weeks.

5. Cytokines should be reconstituted with a carrier protein, such as bovine serum albumin, to maintain stability and stored at −80 °C in high concentrations. Aliquots can then be defrosted prepared in working concentration and either stored short term at −20 °C for maximum 4 weeks or at 4 °C for up to a week.

6. All antibodies used are titrated to give optimal staining and usually the quantity needed is much lower than the amount suggested by the supplier. This allows for extended usage of the antibody.

7. For cell cycle analysis and ploidy the use of peripheral blood as a control for the 2 N peak to configure the flow cytometer for the first time. Also allows easier identification of the ploidy Mks.

8. Platelets generated from umbilical cord blood can be used as a positive control for all experiments testing the platelets produced in culture (6). This makes it much easier to configure the flow cytometer and allow better identification of the cells.

Acknowledgments

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A Simple Protocol for the Generation of Cardiomyocytes from Human Pluripotent Stem Cells

Glen Lester Sequiera, Ashish Mehta, and Winston Shim

Abstract

Efficient generation of cardiomyocytes from pluripotent stem cells (PSCs) for multiple downstream applications such as regenerative medicine, disease modeling, and drug screening remains a challenge. Cardiomyogenesis may be regulated in vitro by a controlled differentiation process, which involves various signaling molecules and extracellular environment. Here, we describe a simple method to efficiently generate cardiomyocytes from human embryonic stem cells and human induced pluripotent stem cells.

Keywords: Pluripotent stem cells, Differentiation, Small molecules, Cardiomyocytes

1 Introduction

The advent of human pluripotent stem cells in 1998 and subsequently reprogramming of somatic cells back to pluripotency have paved way to unparalleled understanding of human biology (1, 2). These human pluripotent stem cells (hPSC), including human embryonic stem cells (hESC) as well as induced pluripotent stem cells (hiPSC) proliferate indefinitely in undifferentiated state and can be differentiated to various somatic cell types, including cardiomyocytes for vast array of applications like regenerative medicine, disease modeling, and drug development. We have previously shown that reprogramming fibroblasts with episomal-based methods could generate hiPSCs, though with low efficiency (3, 4). These hiPSC could be differentiated into cardiomyocytes that show most of the classical cardiac markers. Furthermore, these generated cardiomyocytes show functional characteristics which could be defined by their apt responses to pharmacological agent, showing that these myocytes may have a significant role in drug discovery (5). However, one of the major bottleneck in cardiac research has been the inefficiency of differentiation towards cardiac fate. EB-based methods are more widely used not only for their simplicity, but also they allow proper mesoderm–endoderm cell interactions that are critical for efficient cardiac differentiation (6). Although there are multiple protocols in the literature, their

reproducibility across multiple cell lines and laboratories has been debatable. In recent years, protocols utilizing small molecules to promote cardiac fate commitment have received increasing recognition due to their simplicity. We previously demonstrated that using a small molecule, SB203580, was sufficient to generate myocytes (3). Recently, we have modified the existing protocol to optimize it to be efficient for cardiac differentiation. In this protocol, we describe how cardiac differentiation could be achieved efficiently with a simple cocktail of small molecules to guide differentiation of PSCs towards cardiac fate.

2   Materials

2.1   Differentiation Medium Components

1. Differentiation basal medium (EB20): DMEM-F12 (Life Technologies), 20 % fetal bovine serum (Hyclone), 1× Pen Strep (Life Technologies), 1× GlutaMAX™ (Life Technologies), 1× MEM Non-Essential Amino Acids (Life Technologies), 1 mM beta-mercaptoethanol (Sigma).

2. 1 mg/mL Dispase: Add 10 mg of dispase to 10 mL of DMEM-F12 and mix gently. Filter-sterilize (0.22 μm) the solution, aliquot, and store in −20 °C till further use.

3. 5 mM SB203580: Dissolve SB203580 in DMSO, mix well, aliquot, and store in −20 °C till further use (see **Note 1**).

4. 10 mM IWP4: Dissolve IWP4 in DMSO, mix well, aliquot, and store in −20 °C till further use (see **Note 1**).

5. 1 mg/mL Cyclosporine A: Dissolve 10 mg Cyclosporine A (Sigma-Aldrich) in 10 mL DMEM/F12 and mix well. Filter-sterilize (0.22 μm) the solution, aliquot, and store at 4 °C till further use.

2.2   Culturing Materials

1. Tissue culture dishes: 6-well tissue culture dishes.

2. Ultra-low attachment culture dishes: 6-well sterile ultra-low attachment cell culture dishes (Corning).

3. Cell Scraper.

4. 15 mL Centrifuge tubes.

3   Methods

3.1   Initiation of Cardiac Differentiation (Day 0)

1. The culture dish referred to here will be a 6 cm (BD Biosciences) dish and the lines are maintained on feeder-free system (e.g. Matrigel). The confluency of the human pluripotent stem cells (PSC) culture should be >85 % (**Note 2**).

2. Remove all the differentiated areas (preferably constituting less than 5 % of all the cells). They have to be manually scraped off using 1.0 mL Tip points (e.g. Axygen). The scraping of non-differentiated parts should be kept to minimum (**Note 3**).

3. Wash the undifferentiated cells a couple of times to remove all the scraped cells.

4. Prewarm Dispase (1 mg/mL) in water bath set at 37 °C.

5. Add 1 mL dispase to the dish and ensure that the whole plate is covered with the enzyme. Incubate the plate in the CO_2 incubator for 5–7 min. The timing is slightly variable depending on the cell line. Allow the enzyme to act on the colonies till the colonies show curling at their edges.

6. Once the curling of edges occurs, remove dispase and wash the culture dish thrice with prewarmed DMEM/F12 (2.0 mL each time) (**Note 4**).

7. Post-washing, add, 3 mL of EB20 media and grid the colonies with a fine edge (e.g. 10 or 20 μL tip) (**Note 5**).

8. Using a broad cell scraper, scrape the gridded colonies (**Note 6**).

9. Transfer the media containing the pieces into one well of an ultra-low attachment 6-well plate and top up the medium to 5.0 mL with EB20 (**Note 7**).

10. Add 5 μm SB203580 (a p38-MAPK inhibitor) to the medium in the well in the dark. Shake gently to spread the pieces evenly throughout the well. Keep overnight in the incubator at 37 °C with 5 % CO_2.

3.2 Visualization of EB Formation (Day 1)

1. Following 24 h of incubation, observe for round EB formations (**Note 8**).

2. Gently, using a wide-pore pipette (say 25 or 50 mL), pipette the clumps once or twice to remove all the loose pieces as well as dead cells attached to the freshly formed EBs (**Note 9**).

3. Aspirate the medium with care to remove all the dead cells and loose pieces.

4. Add 3.0 mL of DMEM/F12 media.

5. Transfer all the contents to 15 mL conical centrifuge tubes and spin at 60 g for 2 min.

6. The EBs will readily settle. Gently aspirate off leaving the EBs behind.

7. Replenish with 2 mL of EB20 media with 5 μm SB203580 in dark and transfer all the EBs to the same ultra-low attachment well and return to the incubator (**Note 1**).

8. Repeat medium change every day till day 4.
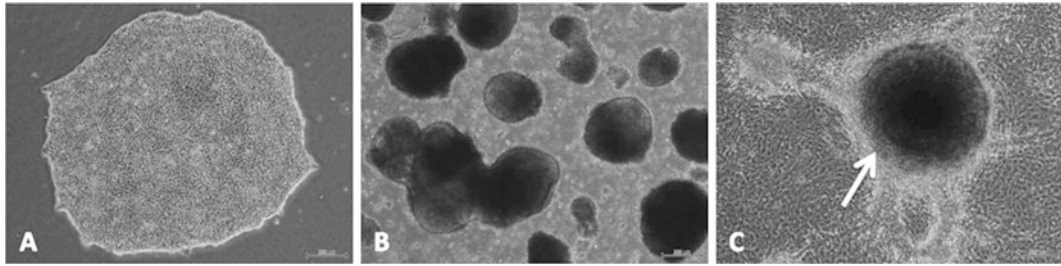
Fig. 1 Stages of cardiomyocyte differentiation. (**a**) Phase contrast image showing an undifferentiated human pluripotent stem cell colony on day 6 of culture appropriate for cardiac differentiation. Scale bar: 200 μm. (**b**) Image showing EBs on day 3 of cardiac differentiation in suspension culture. Scale bar: 200 μm. (**c**) Plated EBs post-day 14 of differentiation showing foci of contracting cardiomyocytes. Arrowhead points towards the beating area. Scale bar: 200 μm

3.3 Induction of Cardiac Specification (Day 4)

1. On day 4 of differentiation, repeat the above steps as indicated till step 6.

2. Replenish with 2 mL of EB20 media with 5μm IWP4 (Wnt Inhibitor) and 3 μg/mL Cyclosporine A in dark. Continue the same step daily till day 8 of differentiation (**Note 1**).

3.4 Plating EBs and Observation of Contractions (Day 4)

1. On day 8 of differentiation, plate the EBs on tissue culture grade plates that have been precoated with 0.1 % gelatin with EB20 medium and return the plates to the incubator.

2. Next day most of the floating EBs would be attached and outgrowths may start appearing.

3. Change the medium with 4 mL EB20 medium and return the plate in the incubator.

4. Observe the culture plates on a regular basis. Contracting EBs may be visible between day 10–14, depending on the particular cell line.

5. The beating clusters can be collected by manual dissection for appropriate downstream applications (Fig. 1 and **Note 10**).

4 Notes

1. SB203580 and IWP4 are light sensitive and thus should be wrapped in Aluminum foil or stored in amber-colored tubes.

2. For PSC lines maintained on the feeders, Collagenase (1 mg/mL in DMEM-F12) may be used instead of dispase for detachment. Avoid incorporation of MEFs while generating EBs.

3. If the differentiated area is more than 5–10 % of the total plate, do not use the plate for cardiac differentiation. Presence of excessive differentiation may significantly hamper differentiation abilities.

4. Colonies post-dispase treatment are fragile and loosely attached. Hence it is recommended that removal of dispase and washing with DMEM-F12 be performed gently. There could be excessive loss of undifferentiated cells at this stage, reducing the number of colonies for cardiac differentiation.

5. Using a tip point, with fine edges (e.g. a 10 μL or a 20 μL tip), to grid the dish is good. However, care should be taken not to damage the colonies with excessive force. If the colonies are very thick and multilayered, a 10×10 grid would be optimal. On the other hand if the colonies are very thin and unilayered, a 6×6 grid will be more favorable.

6. While scraping the gridded colonies with the scraper, try to lift the grids slowly so that the gridded clumps do not break. Breaking the clumps to smaller sizes may significantly hamper EB formation.

7. An acidic pH, denoted by yellowing of the media, leads to cell death in the EB. It is very critical not to allow the medium to become acidic. Medium should be immediately replaced.

8. If EB formation does not occur, the undifferentiated colonies may be allowed to grow for a couple of days more beyond their passage day and thus enabling them to become thicker. However, highly differentiated colonies (>20 %) should be avoided for EB formation.

9. Use wide-bore tips or 25 mL pipettes for this step to avoid sheering of the freshly formed EBs.

10. Typically for a successful differentiation, one can observe about 80–95 % EBs contracting with about 40–60 % cardiac troponin positive cells. This variability is to some extent dependent on the hESC or hiPSC of cell lines' internal abilities towards cardiac commitment.

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Erratum to: Microgrooved Surface Modulates Neuron Differentiation in Human Embryonic Stem Cells

David Lu, Chi-Shuo Chen, Chao-Sung Lai, Sushant Soni, Taranze Lam, Clarence Le, Eric Y.-T. Chen, Thien Nguyen, and Wei-Chun Chin

Erratum to: Methods in Molecular Biology
                 DOI 10.1007/7651_2014_81

The spelling of the name [Taranze Lam] name is incorrect. The name should read Teranze Lam.

The online version of the original chapter can be found under
DOI 10.1007/7651_2014_81

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