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Kursad Turksen *Editor*

Embryonic Stem Cell Protocols

Third Edition

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Edited by

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Preface

Research on embryonic stem cells continues to move very quickly, as reflected in the positive responses that I received for the chapters in this volume, which builds upon a relatively recent previous volume. The kinds of studies continue to expand and diversify, and methodologies are continuously being refined and improved. Amongst areas of intense activity are those related to the very early commitment of stem cells to particular lineages and progression of differentiation to mature cell stages. I am very grateful once again to the contributors who have shared their insights as well as new and improved methods for others already in or just entering the field.

I would like to acknowledge Dr. John Walker, Editor in Chief of the *Methods in Molecular Biology* series, for his continuous support and Patrick Marton, Executive Editor of the series, for encouragement during development of the volume. I am also very grateful to David Casey for being available to answer all of my questions and for providing guidance for shepherding the volume through production.

Ottawa, ON, Canada ***Kursad Turksen***

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Maximizing Clonal Embryonic Stem Cell Derivation by ERK Pathway Inhibition

Jennifer Nichols and Thorsten Boroviak

Abstract

Since the development of inhibitor-based defined culture conditions (known as "2i"), multiple clonal embryonic stem cell (ESC) lines can be readily derived from single cells isolated directly from mouse embryos. In addition to providing an efficient means to generate ES cells from compound transgenic or murine disease models on any genetic background, this technology can be used to investigate the process of ESC derivation at both a functional and molecular level. Here, we provide details of the procedure for both maximizing the number of cells in the donor tissue and subsequent effective derivation of multiple clonal ES cell lines.

Keywords: Blastocyst, Embryonic stem cells, Epiblast, Gsk3 inhibition, ERK/MAPK inhibition, Pluripotency, 2i

1 Introduction

Pluripotency is established during early mammalian development. The preimplantation epiblast represents the founding cell population of the entire fetus. In mouse, this stage of development can be captured in the form of embryonic stem cells (ESCs) (1–3). ESCs functionally contribute to chimeras upon injection into a host blastocyst (4). The unrestricted potential of ESC to give rise robustly to all somatic lineages and the germline has been termed "naïve" pluripotency (5).

ESCs retain their developmental capacity in vitro over extensive time periods when cultured on a feeder layer (1, 2) or in the presence of serum and leukemia inhibitory factor (LIF) (6, 7). More recent work has demonstrated that ESC differentiation is suppressed by the inhibition of the mitogen-activated protein kinase (MAPK) signaling cascade (8). This can be exploited for a defined ESC culture regime, termed 2i, using the Mek inhibitor PD0325901 (PD03) to block the ERK pathway together with glycogen-synthase kinase 3 inhibition by CHIR99021 (CHIR) (9). Addition of LIF is beneficial for clonogenicity, but not required for the maintenance of naïve pluripotency. In practice, any two components of PD03,

CHIR and LIF in serum-free N2B27 medium are sufficient to maintain germline competent ESC (10–12). PD03 mainly represses *Fgf4*-dependent auto-induced differentiation (9), while CHIR and LIF positively stimulate the naïve pluripotency transcription factor circuitry. CHIR stabilizes expression of *Esrrb*, *Klf2*, and *Nanog* via de-repression of *Tcf7l1* (also known as *Tcf3*) (11, 13) and LIF activates the Jak/Stat pathway, resulting in upregulation of pluripotency factors, including *Klf4* and *Tfcp2l1* (14–16). ESCs in 2i/LIF are relatively homogeneous in morphology and pluripotency marker expression, exhibit minimal expression of early lineage specifiers (9, 17) and correspond to the preimplantation epiblast (18). Importantly, 2i/LIF has led to the establishment of germline competent ESC from nonpermissive mouse strains and rats (19–22).

Here, we describe how to maximize mouse ESC derivation efficiency in defined conditions. Our protocol can be applied for routine ESC derivation but is particularly useful when ESC have to be generated from recalcitrant backgrounds. ESC derivation from single cells provides a powerful tool to assess functionally the integrity of individual epiblast cells of mutant or pharmacologically treated embryos. We also describe techniques instructing how to keep track of individual embryos during immunosurgery and subsequent ESC derivation. This can be used for functional endpoint analysis after time-lapse imaging of preimplantation embryos or genotyping from discarded trophectoderm.

The entire protocol takes approximately 8–11 days and is divided into four consecutive steps: (1) embryo culture in ERK pathway inhibition, (2) immunosurgery, (3) plating of epiblast cells, and (4) passaging primary outgrowths. The first step aims to enhance preimplantation epiblast development by FGF signaling inhibition (23). At the 8-cell stage, the embryo undergoes compaction and polarization. In subsequent divisions, the outer cell layer is directed towards the trophectoderm lineage, a prerequisite for blastocyst formation. Interior cells become inner cell mass (ICM) and gradually diverge into pluripotent epiblast and extraembryonic endoderm. In the early ICM (embryonic day (E) 3.25–3.5), cells initially coexpress the early epiblast marker *Nanog* and primitive endoderm marker *Gata6* (24) and the transcriptome of individual early ICM cells is indistinguishable (25). At around the 64-cell stage (E3.75), marker expression becomes heterogeneous and lineages progressively segregate until the late blastocyst stage (E4.5), when the epiblast and primitive endoderm become irreversibly committed (26). In mouse, this process is mediated by FGF signaling (27, 28). Presumptive epiblast cells express *Fgf4*, which reinforces *Gata6* and *Fgfr2* expression in neighboring cells destined to become primitive endoderm (25, 27–29). Pharmacological inhibition of FGF signaling blocks primitive endoderm formation and the vast majority of ICM cells acquire

epiblast identity (23, 30). This provides an ideal starting point for ESC derivation. Embryos cultured in a combination of PD173074 (PD17), a pan-Fgf receptor, and PD03 can give rise to an average of 21 clonal ESC lines each (18).

In the second step of the protocol, the outer trophectoderm is removed by immunosurgery (31). For this technique, blastocysts are incubated with antimouse antibodies. Since trophectoderm is a tight epithelium, epiblast cells residing inside are protected from binding. Subsequently, embryos are washed and exposed to serum with an intact complement system. This results in specific lysis of the antibody-bound outer cells. The isolated ICM consists of enriched preimplantation epiblast, which is then plated out for ESC derivation (Section 3.2.1, step 3). This can either be achieved using whole epiblasts or after single cell dissociation for clonal ESC generation. The last step of the protocol comprises the first passage of primary ESC colonies. This is performed by manual dissociation with a mouth-controlled, finely drawn pipette to ensure minimal carry-over of trypsin and transfer of all dissociated cells from the epiblast. From this step onwards, the cells can be regarded as an ESC line and subsequent passages are carried out by standard tissue culture techniques for further expansion.

2 Materials

2.1 Reagents

1. M2 medium (Millipore).
2. Blastassist medium (Origio) OR KSOM (Millipore).
3. Leukemia inhibitory factor.
4. dH_2O.
5. Ethanol.
6. HCl.
7. PBS.
8. DMSO.
9. PD0325901 (Mek inhibitor).
10. PD173074 (pan-Fgf inhibitor).
11. CHIR99021 (GSK3 inhibitor).
12. N2B27 medium (StemCells, Inc.), ready to use OR home-made N2B27 composed of Insulin, Apo-transferrin, Progesterone, Putrescine, Sodium Selenite, BSA, B27 supplement (Invitrogen), L-glutamine, Neurobasal™ Medium (Invitrogen), DMEM/F12 (Invitrogen).
13. Tyrode's solution, acidic.
14. Rabbit antimouse antiserum.
15. Rat serum, not heat inactivated OR complement sera from guinea pig.

16. Gelatin (Sigma).
17. Fibronectin (Millipore).
18. Laminin511 (Biolamina).
19. Chick serum (Sigma).
20. Trypsin (Invitrogen).
21. EDTA (Invitrogen).
22. Bacteriological dishes (30, 60, and 100 mm).
23. IVF-dishes.
24. Tissue culture-grade 96-, 48-, 12-, and 6-well plates.
25. Dissecting microscope.
26. Humidified incubator at 37 °C with 5–7 % CO_2.

2.2 Recipes

2.2.1 N2B27

In the experience of the authors, N2B27 for conventional or single cell ESC derivation is best bought ready to use from Stemcells, Inc. Home-made N2B27 using either commercially available or entirely home-made supplements (as described below) usually works equally well, however can display some batch variability.

Preparation of Stock Solutions for N2 Supplement

1. Dissolve 100 mg *Insulin* in 4 mL of sterile 0.01 M HCl to give a 25 mg/mL stock solution by incubating overnight at 4 °C. Insulin does not dissolve well, so ensure the suspension is mixed well before aliquoting.
2. Make 100 mg/mL stock of *Apo-transferrin* by incubating 500 mg in 5 mL of dH_2O overnight at 4 °C.
3. Dissolve 6 mg *Progesterone* in 10 mL Ethanol to give a 0.6 mg/mL stock.
4. Dissolve 1.6 g *Putrescine* in 10 mL dH_2O to give a 160 mg/mL stock.
5. Dissolve 2.59 mg *Sodium Selenite* in 5 mL dH_2O to give a 3 mM stock.
6. Dissolve 750 mg *BSA* in 10 mL PBS to make a stock of 75 mg/mL.

Store all stocks at −20 °C.

Preparation of N2 100× Stock

Add the following stocks together: 1 mL *Insulin*, 1 mL *Apo-transferrin*, 0.67 mL *BSA*, 33 μL *Progesterone*, 100 μL *Putrescine*, 10 μL *Selenite*, 7.187 mL *DMEM/F12*.

Preparation of DMEM/F12-N2 Medium

Add 1 mL *N2 stock* to 100 mL DMEM/F12.

Preparation of Neurobasal-B27 Medium

Mix together 1 mL *B27 supplement* (Invitrogen), 1 mL 200 mM *L-glutamine*, 100 mL *Neurobasal*^{TM} Medium.

N2B27 Medium

Mix together DMEM/F12-N2 and Neurobasal-B27 at a 1:1 ratio. Store at 4 °C and use within 2–3 weeks.

2i/LIF (in N2B27)

Add 15 μL 10 mM *CHIR99021*, 3 μL 10 mM *PD0325901*, 5 μL *LIF* (10^7 U/mL) to 50 mL *N2B27*. Store at 4 °C and use within 7 days. In the experience of the authors, addition of antibiotics is not necessary.

2.2.2 Trypsin 1:1

Mix together 0.25 % *trypsin* plus *EDTA* (Invitrogen) and 0.025 % trypsin plus 1 % *chick serum* at a ratio of 1:1.

2.2.3 Fn/Laminin511 Coating Solution

Fibronectin (Millipore) and *Laminin511* (BioLamina) are diluted from frozen stocks to a final concentration of 20 μg/mL in PBS. The solution should be used fresh for best results, but can be stored at 4 °C for up to a week.

2.2.4 Gelatin Coating Solution

0.1 % (weight/volume) of gelatin is diluted in purified water and autoclaved. The solution can be stored at 4 °C for up to 3 weeks.

2.2.5 Glass Capillaries

Quality and size of the glass capillaries used in each step are crucial for handling of embryos, epiblasts or single cells. For this protocol, four different sizes of glass capillaries will be needed: Blastocyst-sized, ICM-sized (two alternative diameters, depending on the level of expansion of the ICM), and cell-sized. Blastocyst-sized glass capilaries should have a diameter of approximately 1.5–2 blastocysts and are best handmade from Pasteur pipettes. ICM-sized capilaries have a diameter of 1.0–1.5 late ICM. For single cell dissociation, it is beneficial to reduce gradually the inner diameter, to a minimum of two single cell lengths. ICM- and cell-sized glass capillaries are best made from microinjection needles produced with a micropipette puller (e.g., Flaming/Brown Micropipette Puller, Model P-97) and a microforge.

3 Methods

The method is summarized diagrammatically in Fig. 1

3.1 STEP1: Embryo Culture with FGF Inhibition

3.1.1 Preparations for Embryo Culture (Day 1)

1. For 10 mM concentrated PD03 and PD17 inhibitor stocks, make a 1:10 pre-dilution in DMSO (1 μM) and add 1 μL of pre-dilution for each inhibitor into 1 mL Blastassist (**Note 1**). Mix well.
2. Take a 60 mm bacteriological dish and pipette an array of 5 × 4 50 μL drops. Do not pipette too close to the walls. Gently add a small amount (100–500 μL) of mineral oil into the dish and ensure that all drops contact the oil. In a second step, add

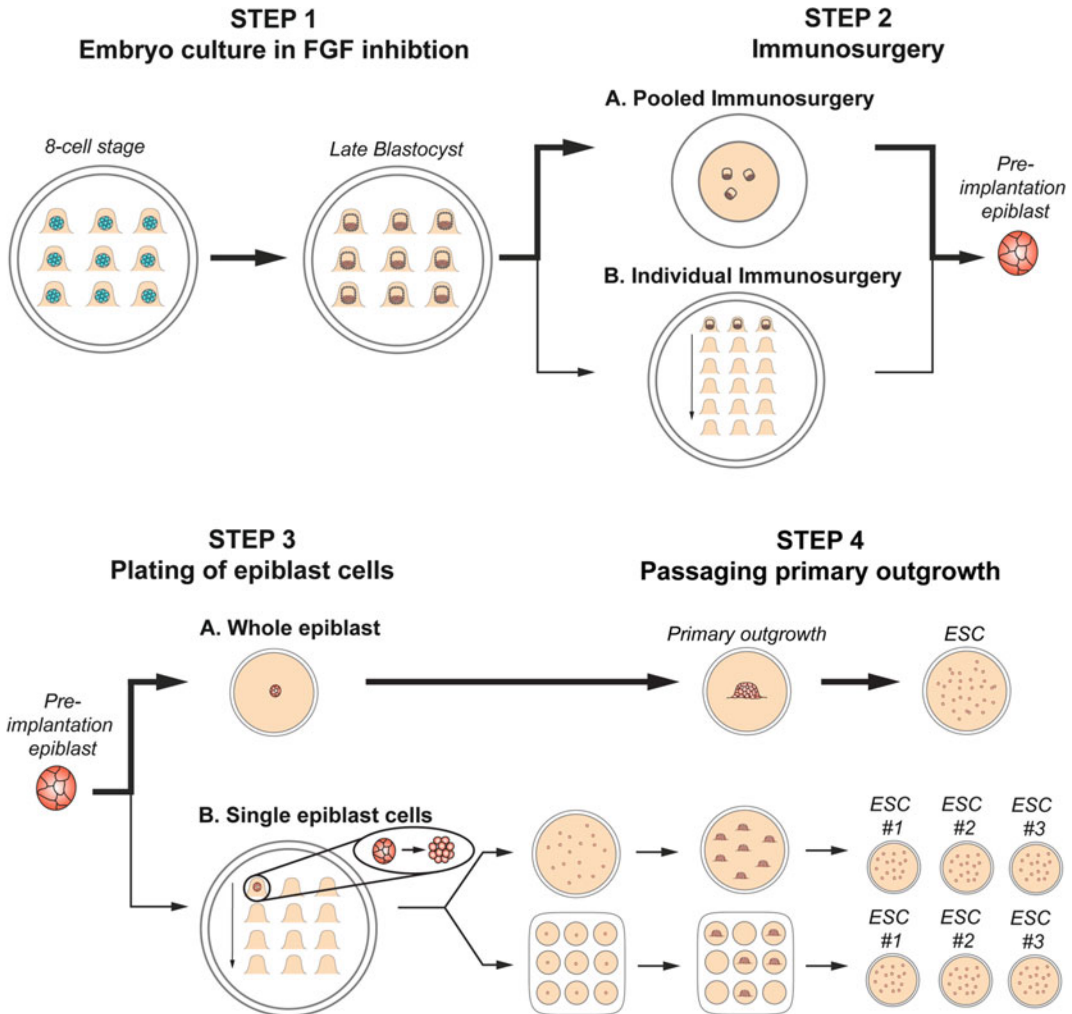

Fig. 1 Diagrammatic representation of the experimental protocol. *STEP 1*: embryos are plated in individual drops of medium (Blastassist supplemented with 1 μM each of PD03 and PD17) under mineral oil, and cultured for 3 days from 8 cell to blastocyst stages. *STEP 2*: Execution of immunosurgery of blastocysts, either pooled or individually to isolate epiblasts. *STEP 3*: Epiblasts are either plated whole, or as single cells separated by trypsinization, in 2i/LIF. Single cells may be scattered within a single well (*top panel*) or individually into micro wells (*bottom panel*). *STEP 4*: Trypsinization and replating of cells following primary outgrowth

another 3–5 mL of mineral oil until all drops are completely submerged (**Note 2**).

3. Place into the incubator for at least 1 h to equilibrate.

3.1.2 Collection of 8-Cell Stage Embryos (Day 1)

1. Flush embryos from oviducts at 8-cell stage (embryonic day (E) 2.5) using a dissecting microscope (**Note 3**) and collect in an IVC dish containing M2 medium using a mouth-controlled finely drawn Pasteur pipette.
2. Transfer isolated 8-cell stage embryos into individual drops in the equilibrated drop-culture dish (Fig. 1, STEP 1).
3. Incubate for 1 day (**Note 4**).

3.1.3 Medium Change for Embryo Culture (Day 2)

1. On day 2, prepare new embryo culture medium. From 10 mM concentrated PD03 and PD17 stocks of inhibitor, make a 1:10 predilution in DMSO (1 μM) for both and add 1 μL of predilution for each inhibitor into 1 mL N2B27. Mix well.
2. Take a 60 mm bacteriological dish and pipette an array of 5 × 4 50 μL drops. Do not pipette too close to the walls. Gently add a small amount (100–500 μL) of mineral oil into the dish and ensure that all drops contact the oil. In a second step, add another 3–5 mL of mineral oil until the drops are submerged.
3. Place into the incubator for at least 1 h to equilibrate.
4. Transfer embryos into the new culture dish (**Note 5**).
5. Incubate for 2 days to allow embryos to reach the late blastocyst stage (**Note 4**).

3.2 STEP 2: Immunosurgery (Day 4)

3.2.1 Preparations for STEP 3: ESC Derivation (Day 4)

1. Coat culture dishes for ESC derivation with gelatin, Fibronectin, or Laminin for 2 h in the incubator (**Note 6**).
2. Aspirate the coating solution and add 2i/LIF without washing.
3. Place into the incubator for at least 1 h to equilibrate.

3.2.2 Immunosurgery (Day 4)

Pooled Immunosurgery

1. Prepare 5 IVF-dishes with 1 mL of N2B27 and 2 with 400 μL N2B27 and pre-equilibrate for about 10 min.
2. Remove zonae pellucidae from any embryos that have not already hatched (**Note** 7).
3. Add 100 μL antimouse serum to one of the 400 μL dishes (20 % final concentration) and transfer the embryos. Incubate for 15 min (Fig. 1, STEP 2).
4. Wash the embryos by taking them through 3 of the 1 mL N2B27 dishes, incubating them for 5 min in each dish.
5. Add 100 μL rat serum (or reconstituted guinea pig complement; **Note 8**) to one of the 400 μL dishes (20 % final concentration) and transfer the embryos. Incubate for 15 min.
6. Using a pipette back-filled with N2B27, transfer the embryos from the complement to a 1 mL N2B27 dish. Incubate for another 15 min to allow for cell lysis in the trophectoderm.
7. Transfer the embryo into a new dish with equilibrated N2B27. Prepare a mouth pipette using with a glass capillary with an inner diameter of roughly the size of an average ICM (~100 μm, Fig. 3: Sizes 2 and 3). Back-fill the pipette and gently aspirate the blastocysts two to three times. The trophectoderm should fall off and reveal the isolated ICM, which should be composed entirely of epiblast cells.

Individual Immunosurgery

1. Pre-equilibrate about 5 mL of N2B27 for about 10 min.
2. To keep track of individual embryos during immunosurgery, they are allocated to individual drops (Fig. 1, STEP 2). Add 400 μL of pre-equilibrated N2B27 to100 μL antimouse serum (20 % final concentration). Prepare an array of 5 × 4 drops (30 μL each) in a 100 mm bacteriological dish. The first line of drops consists of the 20 % antimouse serum dilution in N2B27, the next three rows of N2B27 alone.
3. Transfer individual embryos into the first row and incubate for 15 min.
4. Wash the embryos by transferring them to the lower drop, incubate for 5 min and repeat this step twice. The embryos should now be in the lowest N2B27 drops.
5. Add 400 μL of pre-equilibrated N2B27 to 100 μL rat serum (or reconstituted guinea pig complement; **Note 8**; 20 % final concentration). Assemble a 100 mm bacteriological dish as in step 2 above, but substituting complement solution for the antimouse serum.
6. Transfer the embryos into the 20 % diluted rat serum drops and incubate for 15 min.
7. Transfer the embryos with a pipette back-filled with N2B27 from the 20 % diluted rat serum drop to the N2B27drops in the last row. Incubate for another 15 min to allow for cell lysis in the trophectoderm.
8. Transfer the embryos into a new drop array made from equilibrated N2B27. Transfer the embryos, keeping track of their identity. Prepare a pipette using a glass capillary with an inner diameter of roughly the size of an average ICM (~100 μm, Fig. 3: Sizes 2 and 3). Back-fill the pipette and gently aspirate each blastocyst two to three times. The trophectoderm should fall off and reveal the isolated ICM, which should be composed entirely of epiblast cells. If genotyping of embryos is required, collect the trophectoderm from each and ensure that the identity of the embryo is noted (**Note 9**).

3.3 STEP 3: Plating of Epiblast Cells (Day 4)

3.3.1 Plating Whole Epiblasts

1. Using a blastocyst-sized pipette, place one epiblast into each of the prepared wells for ESC derivation.
2. Incubate for 4–7 days then proceed with passaging the primary outgrowth.

3.3.2 Plating Single Epiblast Cells

1. Pre-warm 5 mL of Trypsin 1:1 and 5 mL N2B27-HEPES for 10 min at 37 °C.
2. Prepare a drop array (Fig. 1, STEP 3) for dissociation by pipetting the first horizontal row with 5 × 30 μL drops of Trypsin 1:1. The remaining rows (2–4) consist of 50 μL drops of N2B27-HEPES.

3. Transfer up to five epiblasts into the first row of Trypsin 1:1, placing one into each drop with a mouth-controlled blastocyst-size glass capillary.
4. Incubate for 15 min. Individual cells should become visible and the whole epiblast should appear like a raspberry (Fig. 1, STEP 3, inset).
5. Using a freshly back-filled (N2B27-HEPES) blastocyst-sized pipette, transfer each epiblast individually into the second row of drops. Take care not to dissociate the epiblast during these washing steps.
6. Back-fill the pipette with fresh N2B27-HEPES and transfer all epiblasts individually into the third row.
7. Repeat step 6 so that all epiblasts are in the last row of N2B27-HEPES.
8. Select a preimplantation epiblast-sized glass capillary (Fig. 3: Sizes 2 and 3), back-fill with N2B27-HEPES and pipette each epiblast three to four times to disaggregate it into small clusters of cells.
9. Switch to a cell-sized glass capillary (Fig. 3: Size 1), back-fill with N2B27-HEPES and dissociate cell clusters into individual cells by repetitive pipetting (**Note 10**).
10. Collect all cells from one epiblast with a freshly back-filled cell-sized glass capillary (Fig. 3: Size 1).
11. Plate individual cells, either by equally distributing them in a single coated dish (e.g., 48-well or 30 mm tissue culture grade) to obtain maximal single cell ESC derivation efficiency (**Note 11**). Alternatively, cells can be plated out individually in small multiwell plates (Fig. 1, STEP 4).
12. Incubate for 6–7 days then proceed with passaging individual colonies.

3.4 STEP 4: Passage 1 (Day 8–11)

3.4.1 Preparations for STEP 4

1. Coat dishes for ESC cultures with gelatin, Fibronectin, or Laminin and incubate for 2 h.
2. Aspirate the coating solution and add 2i/LIF without washing.
3. Place into the incubator for at least 10 min to equilibrate.

3.4.2 Passaging Primary Outgrowths or Colonies from Single Cells (Day 8–11)

1. Pre-equilibrate 5 mL of Trypsin 1:1 and 5 mL N2B27-HEPES for 10 min.
2. Prepare a drop array, as for dissociation of epiblasts, by pipetting the first horizontal row with 5 × 30 μL drops of Trypsin 1:1. The remaining rows (2–4) consist of 50 μL N2B27-HEPES drops (**Notes 12** and **13**).
3. Manually pick an outgrowth or colony using a blastocyst-sized glass capillary back-filled with N2B27-HEPES and transfer it into a drop of Trypsin 1:1. Add up to 5 into the first row.

4. Incubate for 10 min. Individual cells should become visible and the colony should resemble a raspberry.
5. Using a freshly back-filled (N2B27-HEPES) blastocyst-sized mouth pipette, transfer the outgrowths or colonies one by one into the second row of drops into N2B27-HEPES. Take care not to dissociate them during the transfer.
6. Back-fill the pipette with fresh N2B27-HEPES and gently transfer each cell clump into the third row.
7. Repeat step 6. All disaggregates should then be in the last row of N2B27-HEPES.
8. Switch to an epiblast-sized capillary (Fig. 3: Size 3), back-fill with N2B27-HEPES and pipette each clump three to four times to break it into small clusters of cells. These can be further reduced in size by swapping to a cell-sized capillary (Fig. 3: Size 1, **Note 14**)
9. Collect all cells with a freshly backfilled cell-sized glass capillary.
10. Transfer the dissociated cells into a new dish or well.
11. Incubate for 3–5 days then passage using conventional chemical dissociation methods (e.g., accutase or trypsin, **Notes 13** and **15**).

4 Notes

1. KSOM can be substituted for Blastassist.
2. Using individual drops of medium under oil facilitates photography and tracking of each embryo throughout the experiment. However, if this level of attention is not required, embryos can be pooled in a single IVF dish containing pre-equilibrated medium in the central well and PBS in the surrounding trough (to prevent evaporation of medium during culture).
3. To flush the embryos, carefully dissect the oviduct away from the ovary and ovarian fat pad by tearing open the *bursa copulatrix* with fine forceps. Use scissors to cut the ovarian connective tissue and the uterus about 2 mm away from the oviduct/uterine junction. Place the oviduct in a drop of M2 (5–10 μL) in a small bacteriological petri dish. Use a fine (30-G) syringe needle whose tip has been blunted by repeated rubbing on a sharpening stone. The ideal flushing needle has the bevel removed so that the opening is perpendicular to the shaft and the outer surface is rounded towards the tip. Use a 1-mL syringe to direct about 0.2 mL of M2 through the oviduct by inserting the needle into the opening of the infundibulum. It may help to direct the needle into this opening by holding it like a pen, then immobilizing the oviduct by gentle pressure via

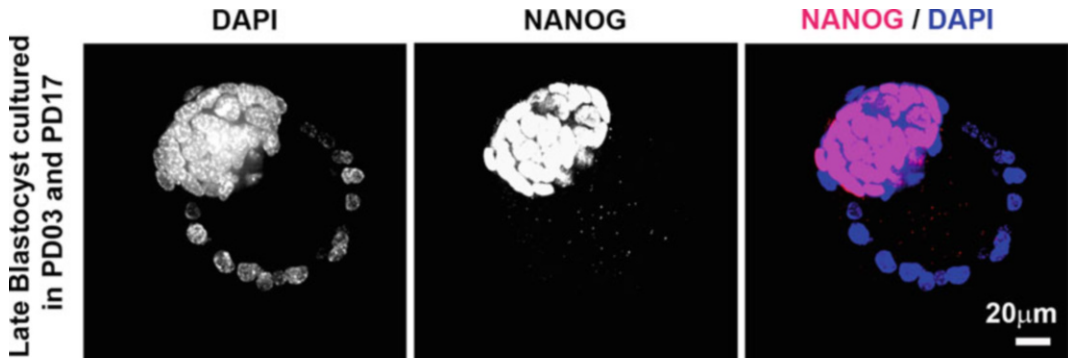

Fig. 2 Confocal image of embryo cultured from 8 cell to blastocyst stage in presence of PD03 and PD17 immunostained for Nanog (*red*) and counterstained with Dapi (*blue*) to show expansion of epiblast

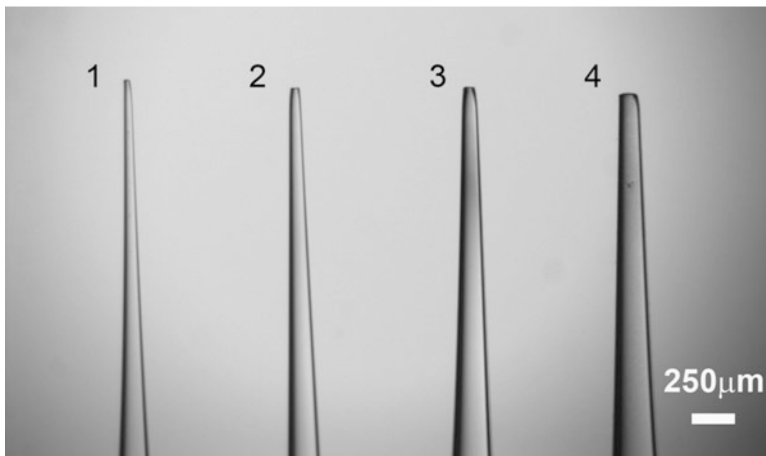

Fig. 3 Photographic examples of the pipettes fashioned for the various stages in the protocol. Size 1 pipettes are suitable for single cells, Size 2 or 3 for ICMs/epiblasts, and Size 4 for whole embryos

the point of the needle to the bottom of the dish whilst the other hand is used to depress the plunger of the syringe. Using a small volume of medium facilitates location and collection of embryos.

4. Incubation in Blastassist or KSOM can be extended to 2 days, if more convenient. The subsequent N2B27 culture period will then be reduced to 1 day. A total of 3 days in culture is optimal. The embryos will then have a large ICM, primarily composed of Nanog-positive epiblast cells (Fig. 2).
5. The embryos should have cavitated by this stage to form early blastocysts.
6. The highest derivation efficiency has been achieved using a mixture of Fibronectin and Laminin511, but the reader may favor the use of gelatin in the interest of cost or convenience.

Microwells, 96 or 48 well plates are ideal for the first plating of separated ICM cells.

7. Place embryos into a drop of acid tyrodes and observe dissolution of the *zona pellucida* under the dissecting microscope. Remove embryos and restore to culture medium as soon as the *zona* has disappeared.
8. If using guinea pig complement, reconstitute with 5 ml sterilized miliQ water, filter, divide into 100 μL aliquots on ice and store at −80 °C. It is essential when using either rat serum or reconstituted guinea pig complement to thaw the aliquot immediately before use. Complement is very unstable and rapidly inactivates when the temperature is allowed to rise.
9. To genotype embryos, collect trophectoderm lysate from each embryo and add individually to 10 μL PCR buffer, incubate at 55 °C for 1 h or O/N (can then be stored frozen until required). Heat at 95 °C for 10 min, centrifuge at 94 g for 10 min, then use 5 μL per PCR reaction (of 30 μL total reaction).
10. It is important to minimize mechanical stress on the cells, so try not to over-pipette them once they have separated.
11. Fibronectin and/or Laminin511 are preferable to gelatin when multiple cells are dispersed within a well since they adhere more rapidly and are less likely to aggregate before attachment.
12. Alternatively, remove medium from each well (observing under dissection microscope to ensure "epiblast" remains in the well) and add a small amount of trypsin, then resuspend in several millilitres of medium, centrifuge at 132 g, aspirate off medium and resuspend into a new 96 or 48 well plate.
13. It is essential to try to remove as much of the trypsin as possible, since the medium contains no serum to neutralize it. Accutase also works well.
14. Dissociating primary outgrowths or colonies into single cells is not necessary; it is usually sufficient to generate a mixture of small clusters and single cells.
15. Freshly derived mouse ESC frequently detach when transferred to gelatin-coated dishes. The authors recommend changing medium with a P-1000 pipette under a dissecting microscope to check that no cells are aspirated. If cells are non-adherent, change half of the medium every day, and collect for passaging by centrifugation.

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A Simple and Efficient Method of Slow Freezing for Human Embryonic Stem Cells and Induced Pluripotent Stem Cells

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Abstract

Protocols available for the cryopreservation of human embryonic stem (ES) and induced pluripotent stem (iPS) cells are very inefficient and laborious compared to those for the cryopreservation of murine ES/iPS cells or other general cell lines. While the vitrification method may be adequate when working with small numbers of human ES/iPS cells, it requires special skills and is unsuitable when working with large cell numbers. Here, we describe a simple and efficient method for the cryopreservation of hES/hiPS cells that is based on a conventional slow freezing method that uses a combination of Pronase/EDTA for Stem™ and CP-5E™ [final concentrations: 6 % hydroxyethyl starch, 5 % DMSO, and 5 % ethylene glycol in saline]. CP-5E™ is highly effective for the cryopreservation of small cell clumps produced by hES/hiPS colony detachment in the presence of Pronase and EDTA (Pronase/EDTA for Stem™, a formulation containing multiple digestive enzymes from *Streptomyces griseus*). This novel method would be quite useful for large-scale hES/iPS cell banking for use in clinical applications.

Keywords: Cryopreservation, Human embryonic stem cells, Human induced pluripotent stem cells, Slow freezing, Hydroxyethyl starch, Dimethyl sulfoxide, Ethylene glycol, Pronase

1 Introduction

Human embryonic stem (hES) cells (1) and human induced pluripotent (hiPS) cells (2) show great potential for use in numerous biomedical applications, including drug development, disease modeling, and cell therapy (3). Regenerative medicine therapies that use hES/iPS are being explored in Japan and the USA for the treatment of retinal diseases such as age-related macular degeneration, Parkinson's disease, and spinal cord injuries (4). Additionally, various iPS cell types have been used to study disease physiology as well as drug targeting. There are, however, several hurdles in the use of hES/iPS cells in cell banking and biomedical applications, including difficulties in the large-scale cryopreservation of hES/iPS cells compared to murine ES/iPS cells or other cells (5–7).

Two methods commonly used for hES/hiPS cell cryopreservation are vitrification (8–11) and slow freezing (12–15). While the vitrification method is adequate for hES/hiPS cell cryopreservation,

it requires special skills and is not suitable for when working with large amounts of cells. In contrast, slow-freezing methods do not require special skills, as they involve cell resuspension in a cryopreservation medium followed by gradual freezing in a deep freezer or programmable freezer, and also allow the researcher to work with large cell numbers. However, the distinct drawback of their use is that the post-thaw recoveries are low compared to those after vitrification. Until now, several approaches to improve efficiency have focused on the use of various cryoprotectant agents (CPAs) (16) and the use of an anti-apoptotic reagent (the Rho-associated kinase (ROCK) inhibitor, Y-27632) (17).

Recently, we developed a novel slow-freezing method using an animal component-free and protein-free cryopreservation medium, termed CP-5E™ [final concentrations: 6 % hydroxyethyl starch (HES), 5 % DMSO, and 5 % ethylene glycol (EG) in saline] (18). This simple formulation minimizes the risk of exposure to xenogeneic pathogens and eliminates the issues arising from lot-to-lot variations in bovine serum albumin (BSA). The use of CP-5E™ is highly effective in the cryopreservation of small clumps prepared by hES/hiPS colony detachment by the use of Pronase (a mixture of proteases originally isolated from *Streptomyces griseus* (19) cultures) in combination with EDTA in the formulation "Pronase/EDTA for Stem™." Using this method, post-thaw recovery frequencies of hES/hiPS cells were above 80 % with retention of the typical cellular morphology. Moreover, the cells exhibited a good expansion profile, were positive for pluripotent markers, and could differentiate into the three germ layers.

CP-5E™ contains both high- and low-molecular weight CPAs. HES is a plant-derived high molecular weight CPA that cannot enter cells and remains in the extracellular space to participate in cell dehydration and minimize intracellular ice crystal formation, thus helping in membrane stabilization (20). This CPA has been used as a plasma volume expander and drug stabilizer, suggesting its biological safety. In contrast, DMSO and EG are low molecular weight CPAs (78.13 Da and 62.07 Da, respectively), and can penetrate the cellular membrane and prevent the formation of ice crystals during cooling or warming.

Our method has the following advantages:

1. Cell detachment with Pronase/EDTA for Stem™ is rapid and can be accomplished in less than 5 min.
2. The reagents are not complex in formulation and are relatively inexpensive.
3. The freeze–thaw method used is simple and does not require intensive training.
4. There is no need for a programmable freezer.
5. Rapid thawing in a water bath is simple and does not require any special post-thaw recovery solutions.

Because the conventional slow freezing method is quite familiar, this easy and robust cryopreservation method can be used widely for basic research and in clinical applications as well.

2 Materials

All reagents and materials used must be sterile.

2.1 Reagents

1. Dulbecco's modified Eagle's media (DMEM)/F12 + GlutaMax™ (Life Technologies, cat no. 10565-018).
2. KnockOut™ serum replacement (KSR: Life Technologies, cat no. 10828-028).
3. MEM nonessential amino acids (100×) (Life Technologies, cat no. 1140-050).
4. 2-Mercaptoethanol (Wako, cat no. 139-06861, Japan).
5. Penicillin–Streptomycin, Liquid (100×) (Life Technologies, cat no. 15140-122).
6. Basic fibroblast growth factor (bFGF) (Wako, cat no. 068-04544, Japan).
7. DMEM, high glucose, pyruvate (Life Technologies, cat no. 11995-065).
8. Fetal Bovine Serum (Tissue Culture Biologicals, cat no. 101, USA).
9. PBS (Phosphate Buffered Salts) tablets (TaKaRa, cat no. T900).
10. Mitomycin C (Kyowa Hakko Kirin, Japan).
11. 0.05 % Trypsin/EDTA (Life Technologies, cat no. 25300-062).
12. Gelatin from porcine skin Type A (Sigma-Aldrich, cat no. G1890-100G).
13. 2.5 % Trypsin (10×), no phenol red (Life Technologies, cat no. 15090-046).
14. Collagenase, Type IV (Life Technologies, cat no. 17104-019).
15. $CaCl_2$ (Wako cat no. 039-00475, Japan).
16. Y-27632 (Wako, cat no. 253-00513, Japan).
17. VECTOR Alkaline Phosphatase Substrate Kit IV (Vector laboratories, cat no. SK-5400).
18. Pronase/EDTA for Stem™ (Kyokuto Pharmaceutical Industrial, cat no. 28111, Japan).
    (a) 0.075 mg/mL Pronase and 0.2 mM EDTA in D-PBS (-) (*see* **Note 1**).

19. Freezing media CP-5E™ (Kyokuto Pharmaceutical Industrial, cat no. 27203, Japan).
    (a) 6 % (w/v) Hydroxyethyl starch, 5 % (v/v) dimethyl sulfoxide, and 5 % (v/v) ethylene glycol in physiological saline.
20. Human ESC line, KhES-1 (Riken BRC, Japan).
21. Human iPS cell line, 201B7 (Riken BRC, Japan).
22. Mouse fibroblast SNL76/7 feeder cell line [an STO cell line that expresses both G418 resistance and leukemia inhibitory factor, European Collection of Cell Culture (ECACC), UK].

2.2 Reagent Setup

1. Human ES/iPS media
   DMEM/F12 containing 20 % KSR, 1 % nonessential amino acids, 0.1 mM 2-mercaptoethanol, 1× penicillin–streptomycin, and 5 ng/mL bFGF: To prepare 500 mL of this media, mix 100 mL KSR, 5 mL of the 100× nonessential amino acid solution, 0.5 mL of 0.1 M 2-mercaptoethanol, and 5 mL of 100× penicillin–streptomycin, and then make up to 500 mL with DMEM/F12. Add 0.5 mL of 5 μg/mL bFGF before use. Store at 4 °C for up to a week.
2. SNL media
   DMEM (high glucose, pyruvate, 2 mM L-glutamine) containing 10 % fetal bovine serum, 1× MEM-NEAA, and 1× penicillin–streptomycin: To prepare 500 mL of this media, mix 50 mL of FBS, 5 mL of 10× MEM-NEAA, and 5 mL of 100× penicillin–streptomycin. Store at 4 °C for up to a week.
3. Gelatin-coated culture dishes
   To prepare a 0.1 % gelatin solution, dissolve 0.5 g gelatin powder in 500 mL of distilled water and sterilize by autoclaving. To coat a culture dish, add a sufficient volume of this solution to cover the bottom of the culture well. For example, 1 mL is required for a 35-mm (6-well plate) surface, while 5 mL is required to coat a 100-mm dish. After coating, incubate the dish for at least 30 min at 37 °C. The excess gelatin solution should be aspirated before use.
4. CTK dissociation solution
   D-PBS(-) containing 0.25 % trypsin, 1 mg/mL collagenase IV, 20 % KSR, and 1 mM $CaCl_2$.
5. ROCK inhibitor Y-27632
   Dissolve 5 mg Y-27632 in 1.48 mL of distilled water to give a 10-mM stock solution. Aliquot and store at −20 °C.

2.3 Equipment

1. Cryovial (2-mL tube, AGC Techno Glass, Japan).
2. 15 and 50 mL conical tubes (Thermo Scientific).
3. 6-well culture plate (BD Biosciences, cat no. 353046).
4. 10 and 25 mL plastic disposable pipettes (BD Biosciences).

5. 0.22-μm filter (Millipore).
6. Disposable syringes, 10 and 50 mL (NIPRO, Japan).
7. Centrifuge (TOMY, LC-230, Japan).
8. Inverted phase-contrast microscope (4, 10, 20, and 40× objectives) (OLYMPUS, IX71, Japan).
9. PCV Clean Bench (HITACHI, Japan).
10. Micropipettes (10, 20, 200, and 1,000 μL) (GILSON).
11. Pipette aid (Drummond Scientific Company).
12. Tissue culture incubator (Pharmaceutical Incubator, USA) maintained at 37 °C and 85 % humidity in a 5 % CO_2 atmosphere.
13. Water bath (Thermal ROBO TR-1A, Iuchi, Japan).
14. Hemocytometer (Cell Science & Technology Institute Inc., Japan).
15. Freezing container (NALGENE™ Cryo 1 °C Freezing Container, Nalgene).

3 Methods

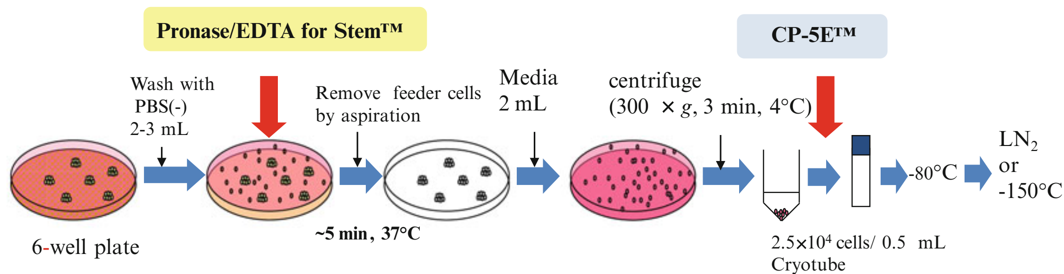
3.1 Passaging of hES/hiPS Cells

Maintain hES/hiPS cells on mitomycin C-treated mouse fibroblast SNL76/7 feeder cells in a gelatin-coated 6-well feeder cell plate. Incubate the cells in a 95 % humidity 5 % CO_2 atmosphere at 37 °C, until they become 80–90 % confluent. Passage colonies by CTK cell detachment treatment every 4 days. Exchange culture media every day except the day following the passaging.

3.2 Freezing Stocks of hES/hiPS Cells

The overall scheme of our protocol is shown in Fig. 1.

1. Grow cells to the exponential phase in a 6-well plate.
2. Aspirate the media and wash the cells twice with 2 mL PBS.
3. Add 1 mL of pre-warmed **Pronase/EDTA for Stem**™ and incubate for 2–5 min at 37 °C (*see* **Note 2**) (Fig. 2).
4. Aspirate **Pronase/EDTA for Stem**™ with the detached feeder cells and gently wash the wells with the human ES/iPS media.
5. Add 1 mL of the human ES/iPS media to the plate. Scrape the colonies off using the pipette.
6. Harvest the cell suspension and centrifuge (300 X*g*, 3 min,4 °C).
7. Discard the supernatant and resuspend the cells in 5 mL of the cold **CP-5E**™ freezing media (*see* **Note 3**).
8. Aliquot the cells (0.5-mL aliquots per 2-mL cryovial; a 1/10 split is suitable).



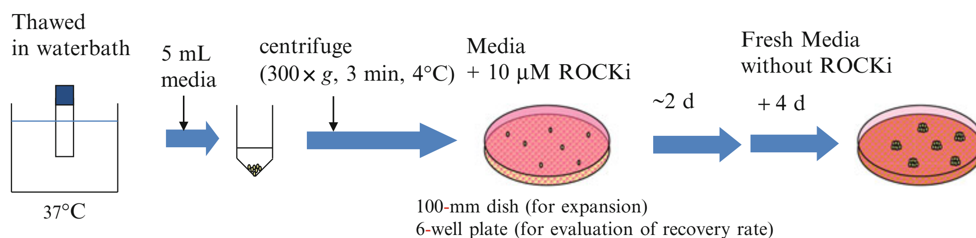


Fig. 1 Schematic showing our cryopreservation protocol, which is based on a conventional slow-freezing method that uses **Pronase/EDTA for Stem**™ and **CP-5E**™. The recovery rate of the cells cryopreserved by this method is more than 80 %, for several hES/hiPS cell lines

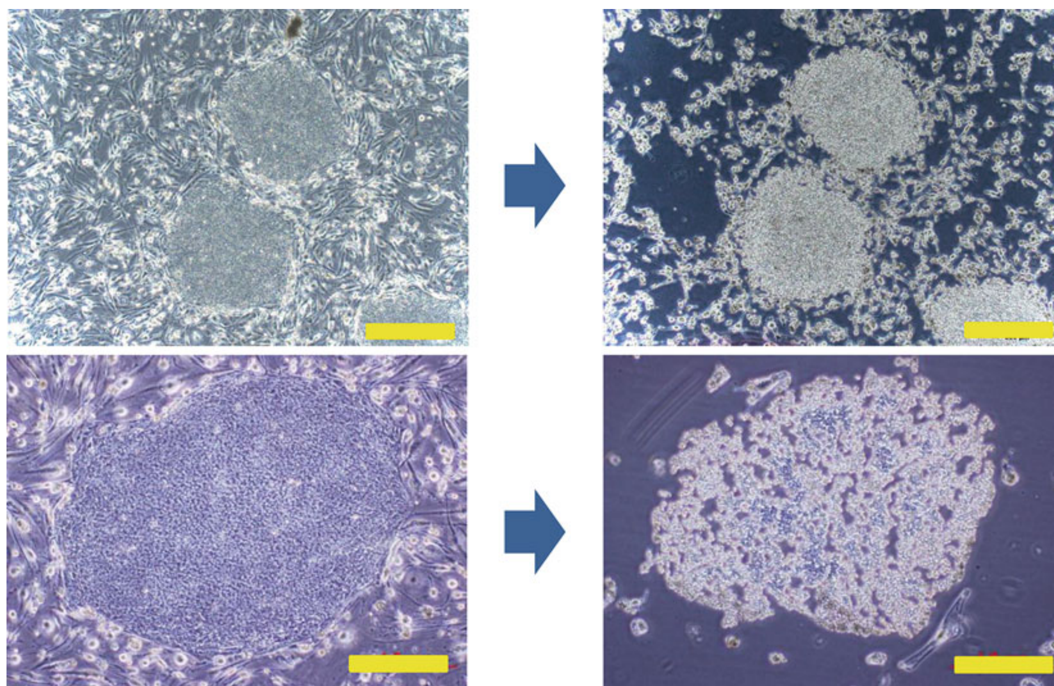


Fig. 2 Detachment of hES/hiPS colonies by Pronase/EDTA for Stem™ Human ES (KhES-1) (*top*) and human iPS (201B7) (*bottom*) colonies before (*upper and lower left panels*) and 3 min after (*upper and lower right panels*) treatment with **Pronase/EDTA for Stem**™. The right-hand side panels show the detachment of the SNL feeder cells and the dissociation of the hES/hiPS colonies. Scale bars indicate 500 µm

9. Place the vials in the cell-freezing container and store at −80 °C overnight.
10. Transfer the vials to liquid nitrogen or −150 °C for long-term storage the following day (*see* **Note 4**).

3.3 Thawing of the hES/hiPS Cells

Preparation of a feeder-seeded culture plate (using one 100-mm dish) 1 day before thawing of hES and hiPS cells.

1. Coat the culture plate with 0.1 % gelatin (10 mL/100-mm dish).
2. Incubate the culture plate at 37 °C for 1 h.
3. Rinse the culture plate with PBS (-).
4. Add the mitomycin C-treated SNL76/7 feeder cells suspended (*see* **Note 5**) in the SNL media to the gelatin-coated dish at a density of 5–7 × 10^3 cells/cm^2 (*see* **Note 6**). Culture overnight. To maximize the viability of the cultured cells, be sure to warm up the media up to 37 °C before use.
5. Draw a vial from liquid nitrogen and immediately thaw in a 37 °C water bath.
6. Remove the vial from the water bath as soon as the cells are thawed and spray with 70 % ethanol to sterilize the surface.
7. Transfer the cell suspension to a 15-cm conical tube containing 5 mL of ice-cold human ES/iPS media and pellet the cells by centrifugation at (300×*g*, 3min, 4°C)
8. Discard the supernatant and resuspend the hES/hiPS cells in 10 mL fresh media containing the ROCK inhibitor Y-27632 (10 μM).
9. After 48 h, remove the media and replace with media containing no ROCK inhibitor (*see* **Note 7**).
10. Incubate at 37 °C, 5 % CO_2 until the hES/hiPS colonies grow to an appropriate size. The media should be changed every day.

3.4 Optional: Alkaline Phosphatase Staining to Verify the Growth of hES/iPS Cells

1. Remove the culture media.
2. Fix the colonies with 4 % (w/v) paraformaldehyde in PBS for 1 h at room temperature.
3. Wash twice with PBS.
4. Start the color reaction using the alkaline phosphatase substrate kit IV, as per the manufacturer's instructions.
5. Stop the reaction after 1 h by washing twice with PBS.
6. Count the stained colonies (Fig. 3).

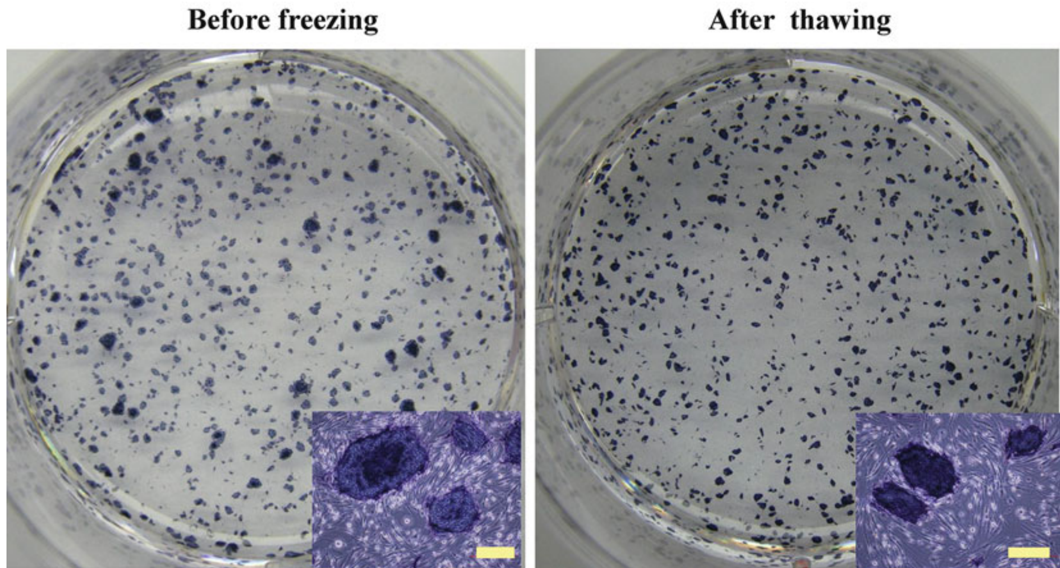


Fig. 3 *Colony formation before freezing and after thawing*. Alkaline phosphatase staining of hiPS 201B7 colonies maintained for 5 days after passage (*left*: post-plating, non-frozen control) and 5 days after thaw (*right*: post-thawing, dissociated using **Pronase/EDTA for Stem**™ and cryopreserved using **CP-5E**™). Magnified photos are attached. Scale bars indicate 500 μm

4 Notes

1. Pronase is isolated from the growth media of *Streptomyces griseus* cultures (19). No animal-derived components are utilized for the culture of this bacterium.
2. The treatment time with **Pronase/EDTA for Stem**™ varies and depends on the cell lines and the quality of colonies used. Cell colonies should not be disrupted into single cells. Pronase dissociates hES/iPS cells by detaching the SNL feeder cells from the hES/hiPS cells, while EDTA breaks the hES/hiPS cell colonies into small clumps. We assume that the small cell clump size (approximately 2,000 μm^2) obtained with the combination of Pronase/EDTA facilitates good delivery of the cryopreservatives to individual cells within the cell clumps.
3. The freezing media **CP-5E**™ should be equilibrated on ice before use.
4. Long-term storage in a −80 °C deep freezer should be avoided, as extended storage at −80 °C causes a decline in cell recovery.
5. The feeder cell density should be determined in advance based on the colony morphology of hES/iPS cells.
6. The mitomycin C-treated SNL dishes should be prepared 1 day before use. In addition, frozen stocks of mitomycin C-treated SNL cells can be prepared using a standard technique and

stored at −80 °C or in a liquid nitrogen tank, in the vapor phase. These stocks should be revived in a gelatin-coated dish or plate within 3 days of preparation.

7. The culture plates should not be moved on the day following the passaging.

Acknowledgements

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Resolving Heterogeneity: Fluorescence-Activated Cell Sorting of Dynamic Cell Populations from Feeder-Free Mouse Embryonic Stem Cell Culture

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Abstract

Embryonic stem cell (ESC) culture comprises a mixture of cells that are primed to differentiate into different lineages. In conditions where ESCs self-renew, these primed populations continuously interconvert and consequently show highly dynamic coordinated changes in their expression of different sets of pluripotency and differentiation markers. It has become increasingly apparent that this transcriptional heterogeneity is an important characteristic of ESC culture. By sorting for specific populations of ESCs it is possible to enrich for cells with a capacity to colonize the embryo proper or the extra-embryonic lineages such as the descendents of the primitive endoderm or trophoblast. Here, we describe a method of isolating specific sub-sets of ESCs from the pluripotent cells present in in vitro ESC culture using SSEA1 antibody staining in combination with reporter lines and fluorescence activated cell sorting (FACS).

Keywords: Embryonic stem cells, Lineage priming, Self renewal, Pluripotency, Endoderm, Transcription

1 Introduction

Embryonic stem cells (ESCs) are characterized by their ability to form any cell type in the adult body (pluripotency) and their capacity to expand indefinitely in culture (self-renewal). These cells are derived from the blastocyst stage of mammalian embryonic development at a point where the first lineage decisions are being made. During early development, the lineage potential of individual cells becomes progressively restricted such that by the late blastocyst stage, three lineage committed progenitor cell types are established: trophectoderm (TE), which will form the placenta, primitive endoderm (PrEn), which will develop into the visceral yolk sac, and epiblast (Epi), which goes on to form the embryo proper (1, 2), including the germ line. The process of specifying these lineages occurs progressively over time, with the TE forming first, at the 8–16 cell stage as an outer epithelial layer that surrounds the inner cell mass (ICM) (3). The specification of Epi and PrEn then becomes apparent at peri-implantation blastocyst stages, from

which most ESC lines are derived. At this stage, the ICM consists of a salt and pepper mix of PrEn and Epi precursors (4–7). These two cell types are marked by the expression of lineage specific transcription factors, including Nanog in the Epi precursors and Gata6 in the PrEn precursors (8). These transcription factors are initially co-expressed in late morula, but become mutually exclusive at E3.5. By E4.5 the Gata6 positive cells form a monolayer of PrEn adjacent to the blastocoel cavity, and the Nanog positive cells form the Epi, which is restricted to the interior of the ICM (8–10). At E3.5, when Nanog and Gata6 become mutually exclusive, individual ICM cells appear committed as lineage tracing experiments show that individual ICM cells at this stage form either Epi or PrEn, but rarely both (9, 11). However, cells from these stages are able to generate both lineages in heterotopic grafting experiments and do not become fully restricted to either Epi or PrEn fate until they have completely segregated and are morphologically distinct at E4.5 (6, 8, 12).

Like the blastocyst, ESC culture is composed of functionally distinct, lineage-primed cell populations that represent precursors of the pluripotent Epi, marked by high Nanog expression (13–15), and the extra-embryonic PrEn, marked by low-level expression of the PrEn marker Hhex in Nanog-low cells (16–18). While the PrEn-primed ESCs don't express Gata6 protein, they do exhibit elevated levels of PrEn RNA expression and have a functional PrEn bias in both in vivo and in vitro differentiation. Thus, ESC culture conditions appear to "trap" cells in a state that is comparable to that in the early blastocyst, as cells are beginning to make their choice between Epi and PrEn. The key to identifying these states is the combination of a marker for PrEn differentiation and a second marker for undifferentiated ESCs. Using this combination of markers, it is possible to sort cells that are both efficiently self-renewing as well as primed for either Epi or PrEn differentiation. In addition to Hhex and Nanog, a number of other markers are also heterogeneously expressed in ESCs (19), including Rex1 (20), Esrrb (21), Dppa3 (Stella) (22), Klf4 and Tbx3 (23), Zscan4 (24), and a two-cell stage specific endogenous retroviral long terminal repeat (25), suggesting that ESC culture contains additional primed populations, although the extent of overlap between these different early lineage markers has yet to be explored.

In this chapter, we provide a detailed description of the techniques involved in separating heterogeneous, but self-renewing, ESC populations. We describe the purification of PrEn- and Epi-primed cells from ESC culture by employing a highly sensitive transcriptional reporter for the PrEn marker Hhex (Hhex-IRES-Venus; HV) (16) and the ESC marker SSEA1 (or PECAM1). An overview of the FACS strategy employed to achieve a clear separation of these populations is shown in Fig. 1.

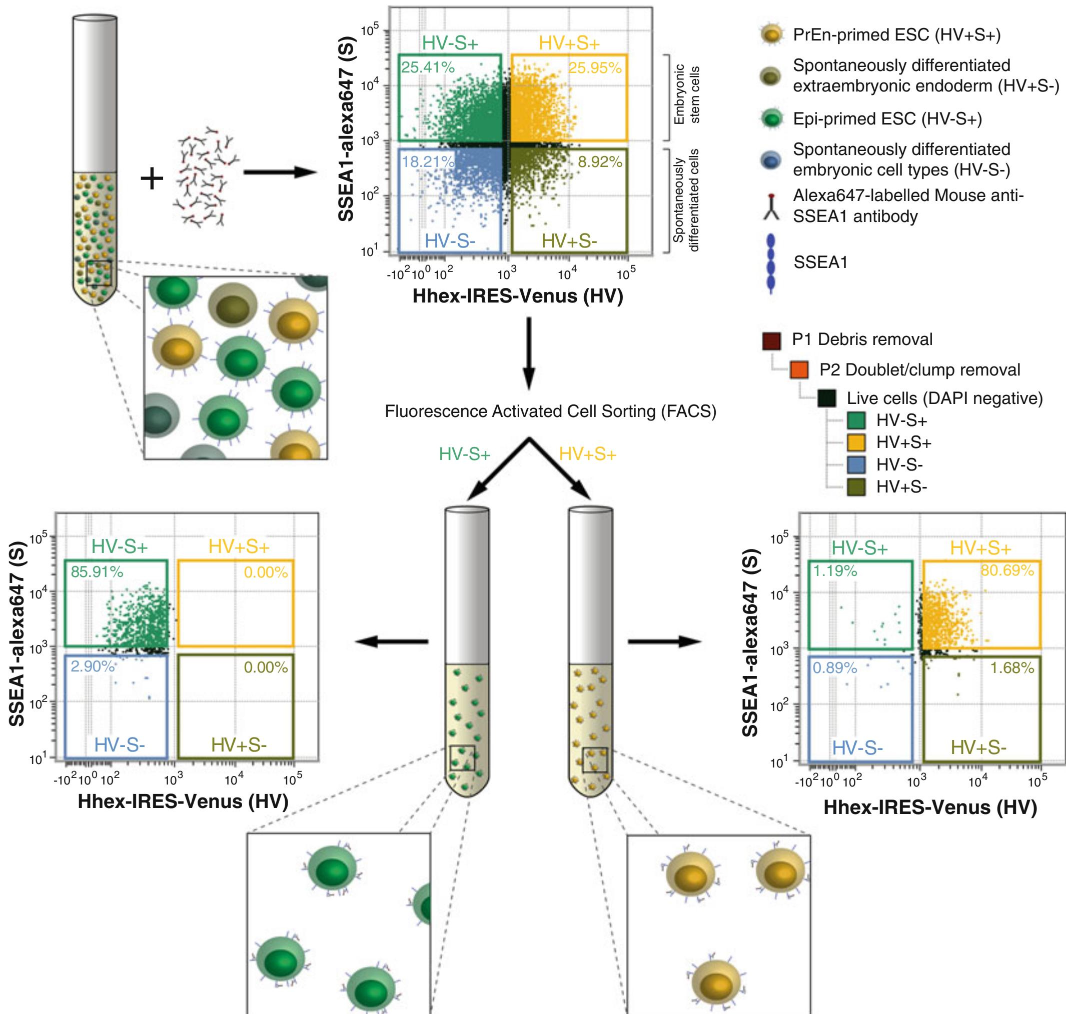

Fig. 1 Schematic overview of the FACS strategy used to isolate primed ESC populations. A single-cell suspension generated from cultured ESCs (*top left*) contains populations of SSEA1$^+$ primed Epi (*green*) and primed PrEn (*yellow*) as well as spontaneously differentiated SSEA1$^-$ embryonic cell types (*light blue*) and extraembryonic endoderm (*dark yellow*). When these cells are stained using a directly conjugated SSEA1 antibody, they can be readily separated in a FACS plot (as shown in the FACS plot at the *top*). Reanalysis of the cells after FACS shows that a large percentage (typically >80 %) of the sorted cells falls within the gate that was used for the respective sorted populations (*bottom half* of the figure). A gating hierarchy (*bottom right*) is used to ensure that the majority of events within the sort gates represent living cells (for details of these gates, *see* Fig. 2)

2 Materials

1. ESCs:
   (a) E14 Tg2a (26).
   (b) HV5.1 (16).

2. ESC medium: GMEM (Sigma G5154), 10 % Fetal Calf Serum (FCS), 1× MEM non-essential amino acids, 0.1 μM 2-Mercaptoethanol, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, and 1,000 U/ml of Lif. Store at 4 °C. Equilibrate to room temperature (RT) or 37 °C before use.
3. Dulbecco's phosphate buffered saline (PBS): 1.34 mM KCl, 0.735 mM KH_2PO_4, 68.4 mM NaCl, 4.05 mM NaH_2PO_4, pH 7.4. Store at RT.
4. Attenuated trypsin: 0.025 % trypsin (Invitrogen 15090-046), 1.27 mM EDTA, 1 % Chicken serum (Sigma C5405) in PBS (suitable for cell culture; without CaCl or MgCl). Store at 4 °C.
5. FACS buffer: 10 % Fetal Calf Serum, PBS (suitable for cell culture; without CaCl or MgCl). Keep on ice when in use and store at 4 °C.
6. 5 ml FACS tubes (Corning catalog nr. 352054).
7. 5 ml FACS tubes with cell strainer cap (Corning catalog nr. 352235).
8. Mouse anti-SSEA1 IgM, κ antibody conjugated to Alexa Fluor® 647 (BD Pharmingen catalog nr. 560120), also *see* **Note 1**.
9. Mouse IgM, κ isotype control antibody conjugated to Alexa Fluor® 647 (0.2 mg/ml; BD Biosciences catalog nr. 560806), also *see* **Note 1**.
10. 30 ml universal tubes (VWR 216-1290).

3 Methods

3.1 Cell Culture

All steps are performed in a laminar flow hood.

Prior to starting work in the laminar flow hood, decontaminate it using 20 min UV exposure (usually a built-in UV lamp) and spray the inside of the hood with 70 % ethanol so that all surfaces inside the hood are decontaminated.

Anything that is transferred into the laminar flow hood is sprayed extensively with 70 % ethanol, including (gloved) hands.

Brightfield microscopy on ES cells is done at either 40 or 100× magnification (generally, a 4× or 10× objective on top of 10× internal magnification is used to obtain this level of magnification).

3.1.1 Thawing Cells

1. Check the number of cells in the vial you intend to thaw from the liquid nitrogen and prepare an appropriately sized plate or flask. Normal plating density (5×10^4 cells/cm^2) does not apply as the level of cell death is much higher for cells frozen in liquid nitrogen.
   (a) 0.5–1.5 × 10^6 cells/vial → thaw in one well of a 6-well plate.

   (b) 1.5–4.5 × 10^6 cells/vial → thaw in a 25 cm^2 flask (T25).
   (c) >4.5 × 10^6 cells/vial → thaw in 75 cm^2 flask (T75).

   Note: if the vial to be thawed is very old or has been stored at −80 °C for a few months, increase cell number/cm^2 to compensate for increased cell death.

2. Coat the flask or plate with 0.1 % gelatin in PBS (~200 μl/cm^2) for at least 5 min at RT.
3. Put the cryovial containing, the cells in ES medium with 10 % DMSO, in a hot water bath at 37 °C until ~ 50 % of the vial has liquefied, then transfer the cryovial into a laminar flow hood.
4. Using a 1 ml pipette, transfer all liquid from the cryovial into a universal tube containing 9 ml of ES medium (at a temperature between RT and 37 °C).
5. Transfer ~500 μl warm ES medium to the cryovial to liquefy the remaining solidified DMSO and then transfer all liquid into the same universal, repeat this step until the entire content of the cryovial has been liquefied and transferred into the universal tube.
6. Pipette up and down with ~50 % volume ~15–20× using a 10 ml pipette to ensure the cells are in single-cell suspension.
7. Centrifuge at 330 × *g* for 3 min at RT.
8. Remove the supernatant (preferably using a vacuum pump to prevent cell loss as the pellet is quite delicate).
9. Resuspend in 400 μl/cm^2 by pipetting up and down with ~50 % volume ≥20× using a 10 ml pipette to ensure the cells are in single-cell suspension.
10. Transfer this single-cell suspension into the appropriate culture vessel.
11. Directionally shake the culture vessel to evenly distribute the cells across the surface:
    (a) Quickly move the culture vessel forwards and backwards five times in a row and then immediately switch to quickly moving the culture vessel from left to right and back again five times in a row.
    (b) Repeat the previous step at least three times.
    (c) Check the culture vessel under a microscope to make sure that the cells are distributed evenly across the surface and if not, repeat the directional shaking until the distribution of cells is even.
12. Transfer the culture vessel to a 37 °C incubator (5 % CO_2, 95–98 % humidity).
13. Tap the culture vessel and change the culture medium after 1 day to remove dead cells, then change the culture medium every other day.

14. Grow the cells until the culture vessel is 60–95 % confluent (this should take ~1–3 days using the densities indicated above).
15. Proceed with passaging as described in the next section.
16. Passage the cells at least three times before starting any experiments to ensure that the cells have recovered from the stress of thawing and have adjusted to the culture conditions.

3.1.2 Passaging Cells

1. Check the culture vessel under the microscope and proceed with passaging if the confluency is 60–95 %.
2. Wash the culture vessel with 400 μl/cm^2 PBS at RT.
3. Aspirate PBS and add 40 μl/cm^2 attenuated trypsin.
4. Shake the culture vessel to spread the trypsin across the entire surface area.
5. Incubate at 37 °C for 2–4 min.
6. Vigorously tap the flask 20–30× to release the cells from the plate and reduce cell clump size.
7. Inactivate the attenuated trypsin by adding 9 volumes of ES medium.
8. Transfer the cell suspension to a universal tube and pipette up and down with ~50 % volume ≥20× using a 10 ml pipette to ensure that the cells are in single-cell suspension (if in doubt, check the level of dissociation under the microscope).
9. Centrifuge at 330 × *g* for 3 min at RT.
10. Dilute the cells at a ratio of 1:2 up to 1:10 into the final plating volume (400 μl/cm^2 (=10 ml/T25)) depending on the size of the pellet.

    OR

    Count the cells using your favorite cell counting method (e.g., using a hæmocytometer) and resuspend the cells at a concentration of 1.25×10^6 cells/ml, then dilute the resuspended cells 1:10 into the appropriate volume for plating (400 μl/cm^2; final cell concentration should be 1.25×10^5 cells/ml (=5×10^4 cells/cm^2))
11. Transfer the appropriate plating volume into the prepared culture vessel(s) (Table 1).
12. Directionally shake the culture vessel to evenly distribute the cells across the surface:
    (a) Quickly move the culture vessel forwards and backwards five times in a row and then immediately switch to quickly moving the culture vessel from left to right and back again five times in a row.
    (b) Repeat the previous step at least three times.

Table 1
Area, volume, and cell amounts for seeding and harvesting mouse ESCs on various types of cell culture plastics

Flasks	*Growth area* (cm^2)	*Flask volume* (ml)	*Cell amount for plating*	*Low confluence* (~60 %)	*High confluence* (~95 %)
25 cm^2	25	10	1.25E + 06	2.50E + 06	7.50E + 06
75 cm^2	75	30	3.75E + 06	7.50E + 06	2.25E + 07
150 cm^2	150	60	7.50E + 06	1.50E + 07	4.50E + 07
Dishes	*Growth area* (cm^2)	*Dish volume* (ml)	*Cell amount for plating*	*Low confluence*	*High confluence*
35 mm	9	3.6	4.50E + 05	9.00E + 05	2.70E + 06
60 mm	21	8.4	1.05E + 06	2.10E + 06	6.30E + 06
100 mm	55	22	2.75E + 06	5.50E + 06	1.65E + 07
150 mm	152	60.8	7.60E + 06	1.52E + 07	4.56E + 07
	For each individual well			*Approximate cell yield*	
Multiwell plates	*Growth area* (cm^2)	*Well volume* (μl)	*Cell amount for plating*	*Low confluence*	*High confluence*
96 well (V bottom)	0.38	152	1.90E + 04	3.80E + 04	1.14E + 05
96 well (flat bottom)	0.32	128	1.60E + 04	3.20E + 04	9.60E + 04
48 well	0.95	380	4.75E + 04	9.50E + 04	2.85E + 05
24 well	1.9	760	9.50E + 04	1.90E + 05	5.70E + 05
12 well	3.8	1,520	1.90E + 05	3.80E + 05	1.14E + 06
6 well	9.5	3,800	4.75E + 05	9.50E + 05	2.85E + 06

(c) Check the culture vessel under a microscope to make sure that the cells are distributed evenly across the surface and if not, repeat the directional shaking until the distribution of cells is even.

13. Transfer the culture vessel to a 37 °C incubator (5 % CO2, 95–98 % humidity).

    At a plating cell density of 5×10^4 cells/cm^2, ES cells will need to be passaged approximately every other day.

3.2 Preparing Cells for FACS

FACS buffer is kept on ice as much as possible to try and keep the cells at around 4 °C during the entire procedure.

Every experiment should include a sample of unstained cells that resemble the stained (reporter) cells, such as E14Tg2a ESCs (for reporter lines the parental is usually the best choice).

Before starting, check the minimum volume required to analyze a sample on the flow cytometer used for sorting and adjust the volume of the controls accordingly.

1. Wash the culture vessel with 400 μl/cm^2 PBS at RT.
2. Aspirate PBS and add 40 μl/cm^2 attenuated trypsin.
3. Shake the culture vessel to spread the trypsin across the entire surface area.
4. Incubate at 37 °C for 2–4 min.
5. Vigorously tap the flask 20–30× to release the cells from the plate and reduce cell clump size.
6. Inactivate the attenuated trypsin by adding 9 volumes of ice-cold FACS buffer and transfer the cell suspension to a universal tube.
7. Pipette up and down with ~50 % volume ≥20× using a 10 ml pipette to ensure the cells are in single-cell suspension (if in doubt, check the level of dissociation under the microscope).
8. Count the cells using an aliquot of this cell suspension and leave the rest of the cells on ice.
9. Preparation of antibody solutions used for staining: For every one million cells that are to be stained, prepare 100 μl of 125 ng/ml Alexa Fluor® 647-conjugated Mouse IgM, κ anti-SSEA1 antibody (concentration varies per lot, so check the concentration and calculate the dilution before starting the protocol; the concentration given here is based on the stock concentration of lot # 2237758, which was 50 μg/ml and was diluted at 1:400) and for control cells, prepare 100 μl per million cells of 125 ng/ml Alexa Fluor® 647-conjugated Mouse IgM, κ isotype control antibody (stock = 0.2 mg/ml → 1:1,600 dilution); centrifuge the prepared antibody solutions at max speed (>10,000 × *g*) for 15 min at RT to remove antibody aggregates, then store the solutions on ice in the dark until use (*see* **Note 1**).
10. Centrifuge the cells at 330 × *g* for 3 min.
11. Remove the supernatant and wash the cells by resuspending in ice-cold FACS buffer (~1 ml per million cells), then split the cells into control and stained cell fractions (*see* **Note 2**).
12. Centrifuge the cells at 330 × *g* for 3 min.
13. Remove the supernatant and resuspend in 100 μl per million cells of isotype control or antibody solution (as prepared in **step 9**).
14. Incubate for 15 min on ice.
15. Centrifuge the cells at 330 × *g* for 3 min.

16. Remove the supernatant and wash the cells by resuspending in ice-cold FACS buffer (~1 ml per million cells).
17. Repeat steps 15 and 16 once.
18. Centrifuge the cells at 330 × *g* for 3 min.
19. Resuspend the cells that are to be sorted by repeated gentle pipetting using a 1 ml pipette at 1–1.5 × 10^7 cells/ml in FACS buffer containing 1 μg/ml DAPI and run them through a cell strainer. Resuspend the control cells in FACS buffer containing 1 μg/ml DAPI at 1 × 10^6 cells/ml in 5 ml FACS tubes (to ensure sufficient volume is available for analysis).

3.3 Fluorescence-Activated Cell Sorting Parameters

The parameters given here are based on sorting with a BD FACS Aria III (for laser/filter combinations, *see* Table 2). Ensure that the sorter is set up so that it collects the following parameters: Linear scale—FSC-A, FSC-W, FSC-H, SSC-A, SSC-W, SSC-H; Biexponential scale—DAPI, Venus (or other reporter fluorescent protein, depending on the cell line used and the populations to be collected), APC/Alexa-647 (or other fluorophore conjugated antibody, depending on the (reporter) cell line used and the populations to be collected).

Use a nozzle of at least 100 μm diameter (*see* **Note 3**).

For further details on cell sorting by flow cytometry, *see* ref. (27).

Table 2
Laser/filter/mirror combinations used to detect the indicated dyes

UV dyes	Application	Laser	Filters
DAPI	Identify dead cells	375 nm	450/40
Green/Yellow Dyes			
GFP	Reporter gene expression	488 nm	530/30
FITC	Antibody staining	488 nm	530/30
Venus	Reporter gene expression	488 nm	530/30
Orange/Red Dyes			
RFP	Reporter gene expression	561 nm	582/15
PE	Reporter gene expression	561 nm	582/15
Alexa-568	Antibody staining	561 nm	582/15
mCherry	Antibody staining	561 nm	610/20
Far-Red Dyes			
APC	Antibody staining	633 nm	660/20
Alexa-647	Antibody staining	633 nm	660/20

E14 Tg2a Negative control	E14 Tg2a 1:400 SSEA1-alexa647	HV5.1 Negative control	HV5.1 1:400 SSEA1-alexa647

E14 Tg2a Negative control

Gate name	% of Parent	% of Total
P1	70.02	70.02
P2	95.50	66.88
Live Cells	96.39	64.46
HV-S+	0.01	0.01
HV+S+	0.00	0.00

E14 Tg2a 1:400 SSEA1-alexa647

Gate name	% of Parent	% of Total
P1	64.98	64.98
P2	94.84	61.63
Live Cells	96.25	59.32
HV-S+	65.47	38.84
HV+S+	0.00	0.00

HV5.1 Negative control

Gate name	% of Parent	% of Total
P1	70.85	70.85
P2	94.32	66.83
Live Cells	96.54	64.51
HV-S+	0.00	0.00
HV+S+	0.03	0.02

HV5.1 1:400 SSEA1-alexa647

Gate name	% of Parent	% of Total
P1	66.91	66.91
P2	94.54	63.26
Live Cells	97.88	61.91
HV-S+	21.81	13.50
HV+S+	22.93	14.20

Gating Hierarchy

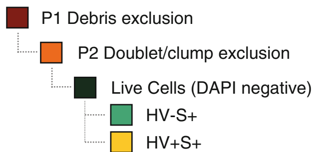

Fig. 2 Gating strategy and requisite control samples. Samples for FACS should always include a negative control (E14 Tg2a negative control) and single positive controls (E14 Tg2a 1:400 SSEA1-alexa647 and HV5.1 negative control) to ensure the gates for the sorted sample (HV5.1 1:400 SSEA1-alexa647) are positioned correctly. Gating forward scatter area (FSC-A) against side scatter area (SSC-A) allows exclusion of cell debris and particulates (the *black dots* to the *left* of P1 in the *dot plots* at the *top*) in the sort tube. Cell clumps can then be removed by gating cells from P1 on forward scatter width (FSC-W) against forward scatter height

3.3.1 Plots

Create the following plots (*see* Fig. 2):

1. Dot plot of SSC-A vs FSC-A.
2. Contour plot with outliers of FSC-H vs FSC-W.
3. Contour plot with outliers of DAPI vs FSC-A.
4. Contour plot with outliers of reporter (Venus) vs. cell surface marker staining (SSEA1-Alexa 647).

3.3.2 Gating

The positioning of gates is determined by including negative control cells (In this case, E14Tg2a stained with Alexa 647-conjugated isotype control IgM at the same concentration as Alexa 647-conjugated SSEA1 (Fig. 2, left column)) and single positive cells (In this case, E14Tg2a stained with Alexa 647-conjugated SSEA1 (Fig. 2, middle left column) and HV5.1 stained with Alexa 647-conjugated isotype control IgM at the same concentration as Alexa 647-conjugated SSEA1 (Fig. 2, middle right column).) to distinguish aspecific staining or autofluorescence from weakly stained cells.

Create the following gates on the plots (numbered as in the Subheading 3.3.1, *see* Fig. 2):

1. Gate on SSC-A vs FSC-A to exclude cell debris and the majority of dead cells (P1 in Fig. 2); select the main population with medium to high FSC-A levels.

   FSC-A measures diffracted light and gives an approximation of cell size while SSC-A measures refracted and reflected light and is indicative of granularity or internal complexity of the detected particle. Cell debris is usually low in FSC-A and dead cells tend to have lower FSC-A and higher SSC-A values than live cells. Applying an initial crude selection based on these parameters simplifies the next gating steps considerably as the position of the gates outlined below is much easier to determine when the majority of unwanted events have been excluded already.
2. In a plot showing only events included in P1, gate on FSC-H vs FSC-W to exclude cell clumps (P2 in Fig. 2); select the main population as identified in a contour plot with outliers.

Fig. 2 (continued) (FSC-H) and excluding events that are larger (i.e., have a bigger FSC-W value) than the main population as defined by the contours in the contour plot with outliers in the *second row* of plots. Finally, dead or dying cells are excluded by including only those events in P2 that are DAPI negative (Live cells gate in the contour plots with outliers shown in the *third row from the top*). This sequential gating strategy ensures that only living single cells are included in the dot plots used to set the sorting gates (*bottom row* of plots). The percentages relative to the parent gate and to the total number of events for each of the gates are shown in the tables at the *bottom*. A schematic overview of the gating hierarchy is shown below the tables

In particular the pulse width (FSC-W) is useful to remove cell clumps as it relates to particle size (28).

3. In a plot showing only events included in P1, gate on DAPI vs FSC-A to exclude dead/dying cells (Live cells in Fig. 2); select DAPI negative cells only.

   Cell impermeant DNA dyes, including DAPI, can enter the cell when membrane integrity is compromised upon necrosis, thus labeling the DNA of dead cells. Note that cells in early phases of apoptosis are not excluded from flow cytometry analysis using this method (29–32).
4. In a plot showing only events included in the Live cell gate, gate on reporter vs. cell surface marker to select the populations of interest (in this case HV^{-} S^{+} and HV^{+} S^{+}, gated to include 20–25 % of the total number of events in the plot). See the gating hierarchy given in Figs. 1 and 2 for an overview of all the gates and how they are related to eachother.

3.4 Collecting Cells After Sorting

1. Collect cells in 5 ml FACS tubes containing 1 ml FACS buffer: 10 % FCS in ice-cold PBS (*see* **Note 4**).
2. Sort up to 750 k cells per tube (when using a 100 μm nozzle; *see* **Note 3**).
3. Keep on ice after sort.
4. Spin FACS tubes at 500 × *g* for 3 min at 4 °C.
5. Coat 1.5 ml Eppendorf tubes with BSA by pipetting 1 ml of 7.5 % BSA (sterile) into the tube, let it sit for 10 s and then wash the tube with 1 ml PBS (the same 1 ml of BSA can be used to coat all tubes needed).
6. Check the tube to see if the cells have pelleted.
7. Partially remove supernatant until ~200 μl is left.
8. Resuspend the cells in the remaining 200 μl and transfer to 1.5 ml BSA-coated Eppendorf tube.
9. Spin at 500 × *g* for 3 min at 4 °C.
10. Check the tube to see if the cells have pelleted.
11. Carefully remove sup (keep an eye on the pellet!) using a P1000 until ~10–20 μl is left, add 1 ml of ice-cold PBS and resuspend pellet by repeated pipetting.
12. Spin at 500 × *g* for 3 min at 4 °C.
13. Check the tube to see if the cells have pelleted.
14. Carefully remove sup (keep an eye on the pellet!) using a P1000 until ~10–20 μl is left, then remove the rest of the PBS using a P200.
15. Resuspend pellet by flicking the tube.

3.4.1 RNA Isolation

1. For RNA add the appropriate lysis and RNA extraction reagent (such as 350 μl buffer RLT (without β-mercaptoethanol) when using Qiagen Rneasy columns or 400 μl TRIzol (Ambion) or TRI reagent (MRC) when using Guanidinium thiocyanate-phenol-chloroform extraction) and ensure cell lysis by repeated pipetting. Leave at room temperature for at least 2 mins and then store at −20 °C or −80 °C.
2. Proceed with RNA isolation according to the manufacturer's protocol.

3.4.2 Protein Isolation

1. For protein add 100 μl/million cells of Laemmli lysis buffer (2 % w/v SDS, 10 % v/v glycerol, 120 mM Tris–HCl pH 6.8).
2. Sonicate on ice (probe):
   (a) 100–250 μl: 10 s at 20 % power.
   (b) 250–500 μl: 15 s at 30 % power.
3. Boil lysates at 95 °C for 5 min.
4. Incubate on ice for 10 min.
5. Spec on Nanodrop using protein 280 setting to get the approximate protein concentration.
6. Store at −20 °C.
7. Just before loading the samples onto a gel, add 10 % v/v 1 M DTT containing bromophenol blue; load 20–60 μg of protein per lane in equal volumes (i.e., all samples in the same volume) of Laemmli (maximum of ~25 μl for standard 10 lane gels).

3.5 Data Presentation

The flow cytometry plots presented in this chapter were generated using FCS Express 4 Flow Research (v. 4.07.0011) and figures were then assembled in Adobe Illustrator CS6 (v. 16.0.3). Plots were copied from FCS express 4 directly into Adobe Illustrator to generate high resolution vector based images. Small size pdfs of the figures were obtained by rasterizing (using "Object > Flatten Transparency...") areas within the figure that contained high vector densities.

4 Notes

1. Alternatively, use 500 ng/ml APC-conjugated PECAM1 Rat IgG2a, κ antibody (1:400 from 0.2 mg/ml; BD Pharmingen catalog nr. 551262) and 500 ng/ml APC-conjugated Rat IgG2a, κ isotype control (1:400 from 0.2 mg/ml; BD Pharmingen catalog nr. 554690). These antibodies are also available with alternative conjugates and their equivalent directly conjugated isotype controls (*see* Table 3). For other conjugates and cell lines with different genetic backgrounds (the lines used here were derived from E14Tg2a 129P2/OlaHsd background

Table 3
Antibodies and isotype controls used to detect self-renewing embryonic stem cells (all from BD Pharmingen)

SSEA1 (CD15)	*Clone*	*Host species*	*Ig subtype*	*Conjugate*	*Catalog nr.*
SSEA1-Alexa-647	MC480	Mouse	IgM, κ	Alexa-647	560120
SSEA1-PE	MC480	Mouse	IgM, κ	PE	560142
SSEA1-FITC	MC480	Mouse	IgM, κ	FITC	560127
IgM isotype control-Alexa-647	G155-228	Mouse	IgM, κ	Alexa-647	560806
IgM isotype control-PE	G155-228	Mouse	IgM, κ	PE	555584
IgM isotype control-FITC	G155-228	Mouse	IgM, κ	FITC	553474
PECAM1 (CD31)	***Clone***	***Host species***	***Ig subtype***	***Conjugate***	***Catalog nr.***
PECAM1-APC	MEC13.3	Rat	IgG2a, κ	APC	551262
PECAM1-PE	MEC13.3	Rat	IgG2a, κ	PE	553373
PECAM1-FITC	MEC13.3	Rat	IgG2a, κ	FITC	553372
IgG2a isotype control-APC	R35-95	Rat	IgG2a, κ	APC	554690
IgG2a isotype control-PE	R35-95	Rat	IgG2a, κ	PE	553930
IgG2a isotype control-FITC	R35-95	Rat	IgG2a, κ	FITC	553929

ESCs (26); see http://www.informatics.jax.org/mgihome/nomen/strain_129.shtml) than the ones described here, the optimal concentration should be determined empirically.

2. Usually, the number of cells needed for a quick analysis of the negative control is ~2×10^5, but if you're working with precious cells, this can be reduced to ~5×10^4. Due to the small volume used for staining cell numbers below the standard 2×10^5 (=20 μl of diluted antibody), staining should be done in a 0.5 ml eppendorf tube to ensure proper mixing of the cells with the antibody.
3. When using a nozzle size bigger than 100 μm, less than 750 k cells will fit into a FACS tube containing 1 ml of buffer. The amount of cells that can be collected in one tube will depend on the size of the nozzle.
4. Survival of cells after the sorting procedure is strongly enhanced by the presence of at least 2 % serum. By adding 1 ml of 10 % FCS in PBS to the 5 ml FACS tube prior to sorting the FCS percentage is always above 2 %. Vortexing the sort tube containing 1 ml of 10 % FCS in PBS prior to starting

the sort also ensures that the sides of the tube are coated with FCS to prevent cells sticking to the side of the tube.

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Acquiring Ground State Pluripotency: Switching Mouse Embryonic Stem Cells from Serum/LIF Medium to 2i/LIF Medium

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Abstract

Mouse embryonic stem cells (ESCs) derive from the inner cell mass (ICM) of a blastocyst. These cells are pluripotent and thus able to generate both somatic and germinal lineages. It is possible to maintain ESCs in different pluripotent states depending on the in vitro culture conditions. Classically, ESCs are cultured in the presence of serum and LIF, which sustain the naive state of pluripotency but in this metastable state cells exhibit a large degree of heterogeneity. In the last few years, it has been discovered that when ESCs are cultured in a chemically defined medium (without serum), in the presence of LIF and with the addition of two small molecules (in particular the inhibitors of MAPK and Gsk-3 pathways), they reach a ground state of pluripotency where cells are more homogeneous and more "ICM-like." In this protocol, we describe how we culture mouse ESCs and the way we switch them from naive to ground state.

Keywords: Mouse embryonic stem cells (ESCs), Serum, 2i, Chemically defined medium (CDM), Laminin

1 Introduction

Mouse embryonic stem cells (ESCs) derive from the inner cell mass (ICM) of an E3.5 embryo at the blastocyst stage (1). These cells are in a pluripotent state that means they are characterized by the ability to differentiate and generate both somatic and germ lineages, indeed when injected into a blastocyst, these cells are able to generate chimeric embryos. In addition, when injected subcutaneously into a mouse, they give rise to teratomas (2). Historically, mouse ESCs were maintained in the pluripotent state by culturing them in fetal bovine serum (FBS) containing medium, on a layer of mitotically inactivated fibroblasts called "feeder" cells, which provides trophic factors for the growth of ESCs (3). In particular, "feeder" cells were shown to produce Leukemia inhibitory factor (LIF) and so the addition of this interleukin into the medium could replace feeders (4). LIF is an activator of the transcriptional factor Stat3, which inhibits ESCs differentiation and promotes self-renewal (5). ESCs cultured in serum/LIF conditions

are defined to be naive pluripotent cells in order to distinguish them from Epiblast stem cells (EpiSCs), which are in a primed pluripotent state, more prone to differentiation (6). EpiSCs derive from late epiblast, a postimplantation mouse embryo at E5.5 and in vitro they required Activin A and Fibroblast Growth Factor 2 (FGF2) instead of LIF and serum to sustain pluripotency (7, 8). Analysis of ESCs cultured in serum conditions showed a strong heterogeneity in the population of cells, even in the expression of pluripotency factors, due to the uncontrolled and multifactorial stimulation by all the extracellular signals present in the serum. The identification of two small molecules that could substitute for serum to sustain ESCs culture was a turning point in the field. In particular, it is possible to culture ESCs without serum with the addition of PD0325901, an inhibitor of mitogen-activated protein kinase (MAPK), and CHIR99021, an inhibitor of glycogen synthase kinase-3 (Gsk3) (9), and in presence of LIF. This new defined medium called "2i" leads to obtain ESCs in a new state: ground pluripotency. ESCs in 2i are more homogeneous and show lower expression of lineage-associated genes and less DNA methylation, so they are closer to ICM-like cells (10, 11). Concerning mouse pluripotency three distinct states can be distinguished: ground, naive, and primed, which correspond to ESCs in 2i conditions, ESCs in serum condition and EpiSCs, respectively. These states are reversible and interconvertible. One of the advantages of using a completely defined medium is that the same basal medium can be used to convert ESC to EpiSC, with the only change being the added factors, in this specific case from 2i/LIF to Activin A/FGF2.

2 Materials

1. Conical centrifuge tubes 15 and 50 mL, sterile.
2. Graduated plastic pipettes (sterile, single package) of 2, 5, 10, and 25 mL.
3. Glass Pasteur pipettes sterilized in an aluminium container using a dry oven (4 h at 180 °C).
4. Plastic sterile petri dishes for cell culture of 35 and 60 mm of diameter.
5. Cryotube vials of 1.8 mL.
6. Freezing container for tube of 1.8 mL (rate of cooling −1 °C/min).
7. Low temperature freezer (−80 °C).
8. Liquid nitrogen container (−196 °C).
9. Pipets P1000, P200, P20, P10, and sterile plastic tips.

10. 20 mL syringes.
11. Syringe membrane filters, 0.22 μm, in PES (Polyethersulfone).
12. Water bath.
13. Centrifuge (for 15 mL plastic tubes).
14. Incubator at 37 °C in a humid atmosphere with 5 % CO_2.
15. Vertical laminar flow hood.
16. Aspiration system.
17. H_2O Milli-Q produced with a resistivity of 18.2 MΩ cm at 25 °C and sterilized.
18. Dimethyl sulfoxide (DMSO) ≥99 %.
19. Dulbecco's phosphate-buffered saline (DPBS) 1× sterile without Ca^{2+} and Mg^{2+}.
20. Trypsin-EDTA (0.25 %). Aliquots of 10 mL stored at −20 °C.
21. Protease-free BSA (Bovine serum albumin). Powder stored at +4 °C.
22. 2-Mercaptoethanol (50 mM). Aliquots of 1 mL stored at +4 °C.
23. Transferrin. Resuspended in H_2O Milli-Q at the final concentration of 30 mg/mL. Aliquots stored at −20 °C.
24. Recombinant Insulin. Resuspended in H_2O Milli-Q at the final concentration of 10 μg/mL. Aliquots stored at −20 °C.
25. Leukemia Inhibitory Factor (LIF). Resuspend in PBS/BSA 0.1 % to the final concentration of 10 μg/mL (10^6 U/mL). Aliquots stored at −20 °C.
26. Laminin 1 mg/mL. Aliquots of 10 μL stored at −20 °C.
27. CHIR99021. Resuspended in DMSO to a final concentration of 10 mM. Aliquots stored at −20 °C.
28. PD0325901. Resuspended in DMSO to final concentration of 10 mM. Aliquots stored at −20 °C.
29. Gelatine Type A from porcine skin. Resuspended at 0.2 % in H_2O Milli-Q and sterilized. Stored at +4 °C.
30. Ham's F-12 Nutrient Mix 1×, supplemented with 2 mM of L-glutamine. Stored at +4 °C.
31. Iscove's Modified Dulbecco's Medium (IMDM) 1×, supplemented with 2 mM of L-glutamine. Stored at +4 °C.
32. Dulbecco's Modified Eagle Medium (DMEM) 1×, supplemented with 2 mM of L-glutamine. Stored at +4 °C.
33. FBS (Fetal bovine serum) tested for ESC culture. Stock stored at −80 °C, while aliquots of 50 mL at −20 °C.
34. FBS (Fetal bovine serum). Stock stored at −80 °C, while aliquots of 50 mL at −20 °C.

35. Chemically Defined (CD) Lipid Concentrate. Aliquots of 10 mL stored at +4 °C.
36. 1-Thioglycerol ≥97 %. Aliquots of 50 μL stored at +4 °C.

3 Methods

All the cell culture work is performed under sterile condition: manipulation of cells and preparation of solutions are done under a vertical laminar flow hood. ESCs are cultured at 37 °C in a humid atmosphere with 5 % of CO_2.

3.1 Serum-Containing Medium

1. For ESC culture, the serum-containing medium is prepared with 85 % DMEM 1× (supplemented with 2 mM of L-glutamine), 15 % FBS tested for ESC culture (*see* **Note 1**), 0.1 mM of 2-mercaptoethanol, and 800 U/mL of LIF. Serum/LIF medium can be kept for 1 month at +4 °C.
2. Inactivating medium: the serum-containing medium to inactivate the Trypsin is prepared with 90 % DMEM 1× (supplemented with 2 mM of L-glutamine), 10 % FBS, and 0.1 mM of 2-mercaptoethanol.

3.2 Chemically Defined Medium (CDM)

1. CDM is prepared with 50 % IMDM 1× (Supplemented with 2 mM of L-glutamine), 50 % Ham's F-12 Nutrient Mix 1× (Supplemented with 2 mM of L-glutamine), 5 mg/mL BSA (*see* **Note 2**), 1 % CD lipid concentrate, 450 μM of 1-thioglycerol, 7 μg/mL recombinant insulin, and 15 μg/mL transferrin. The CDM is then sterilized by filtering with 0.22 μm PES membrane filter. CDM can be kept for 1 month at +4 °C.
2. The final ESC 2i culture medium is prepared by adding 3 μM of CHIR99021, 1 μM of PD0325901, and 700 U/mL of LIF to CDM. CDM/2i/LIF can be kept for 1 month at +4 °C.

3.3 ESC Cultured in Serum-Containing Medium

1. Incubate dishes with Gelatin 0.2 % (1 mL for dishes of 35 mm) for at least 1 h at 37 °C.
2. Pre-warm ESC culture serum-containing medium, Trypsin-EDTA (0.25 %), and inactivating medium in the water bath at 37 °C.
3. Aspirate gelatin from the dishes with a sterile glass Pasteur pipette.
4. Replace the gelatin by serum-containing medium for ESC (1.5 mL for dishes of 35 mm) and put the dishes in the incubator for equilibration.
5. Aspirate the old medium from the dish with cells at confluence.

6. Quickly wash cells with DPBS kept at room temperature (2 mL for dishes of 35 mm).
7. Add Trypsin-EDTA (0.25 %) to cells (1 mL for dishes of 35 mm) and incubate for 2 min at 37 °C to detach cells from the dishes.
8. Add on top the same volume (1 mL for dishes of 35 mm) of inactivating medium and completely dissociate the cells by pipetting several times with a P1000 pipet.
9. Transfer the cells into a 15-mL plastic tube and centrifuge 5 min at 200 × *g* at room temperature.
10. Aspirate the supernatant and resuspend thoroughly the visible cell pellet with fresh ESC culture serum-containing medium by pipetting with P1000 pipet.
11. Finally plate the cells in the new dish (*see* **Note 3**).

3.4 ESC Cultured in CDM/2i Medium

1. Incubate dishes with Laminin (*see* **Note 4**) diluted directly and freshly in DPBS (1 mL for dishes of 35 mm) at the final concentration of 10 μg/mL for at least 1 h at 37 °C (*see* **Note 5**).
2. Pre-warm CDM, CDM/2i/LIF, Trypsin-EDTA (0.25 %), and the inactivating medium in the water bath at 37 °C.
3. Remove Laminin from the dishes by aspiration.
4. Replace Laminin by CDM/2i/LIF medium (1.5 mL for dishes of 35 mm) and put the dishes in the incubator for equilibration.
5. Aspirate the old medium from the dish with cells at confluence (ESC in CDM/2i/LIF or ESC in serum/LIF with or without feeder cells, *see* **Note 6**).
6. Add directly, without DPBS washing step, Trypsin-EDTA (0.25 %) to cells (1 mL for dishes of 35 mm) and incubate for 3–4 min at 37 °C to detach cells from the dishes (*see* **Note 7**).
7. Add on top the same amount (1 mL for dishes of 35 mm) of inactivating medium and dissociate the cells by pipetting with P1000 pipette.
8. Transfer the cells into a 15-mL plastic tube and centrifuge 5 min at 200 × *g*.
9. Aspirate the supernatant and resuspend the visible cell pellet with fresh CDM by pipetting with P1000 pipette in order to wash cells from serum.
10. Centrifuge another time for 5 min at 200 × *g*.
11. Aspirate the surnatant and resuspend thoroughly the visible cell pellet of with fresh CDM/2i/LIF medium by pipetting with P1000 pipette.
12. Finally plate the cells in the new dish (*see* **Notes 8** and **9**).

3.5 Freezing and Thawing ESC

1. Dissociate the cells with Trypsin and prepare a cell pellet as in Sect. 3.3 (**steps 5–9**) or Sect. 3.4 (**steps 5–8**).
2. Remove the supernatant by aspiration and resuspend the visible cell pellet by pipetting with P1000 pipette 1 mL of freezing medium freshly made with 70 % of ESC culture serum-containing medium, 20 % FBS, and 10 % of DMSO for ESC in serum conditions or 60 % of CDM, 30 % FBS, and 10 % of DMSO for ESC in 2i condition.
3. Transfer the cells in freezing medium into a cryotube vial of 1.8 mL and this one into a Freezing container, which is then put for a couple of days at −80 °C.
4. Finally transfer the frozen vials into a liquid nitrogen container (−196 °C) for long-term storage.
5. Pre-warm CDM, CDM/2i/LIF medium, serum/LIF medium, and inactivating medium in the water bath at 37 °C.
6. Thaw the vials with cells in the water bath at 37 °C.
7. Add the 1 mL of cells in freezing medium on top of 4 mL of serum-containing medium for ESC in serum conditions, or CDM for ESC in 2i condition, in a 15-mL plastic tube and centrifuge for 5 min at 200 × *g*.
8. Aspirate the supernatant and resuspend the visible cell pellet of with fresh serum/LIF medium or CDM/2i/LIF medium (according to ESC culture conditions) by pipetting with P1000 pipette.
9. Finally plate the cells in the new gelatin- or laminin-coated dish accordingly (*see* **Note 10**).

4 Notes

1. It is important to test different types of FBS in order to find a batch that leads to optimum cell growth and maintenance of pluripotency of ESCs.
2. It is also necessary to test different batches of BSA for ESC culture to check for optimal growth and absence of differentiation.
3. ESCs in serum-containing medium are usually passaged every 2 days with a dilution of 1/6, if there is a lot of mortality, the medium is changed daily. To let the cells grow for 3 days, dilute them 1/12.
4. ESCs in 2i condition could also be cultured on gelatin-coated dishes, but they will grow as ball-like colonies, sometimes loosely attached and this becomes an issue when performing

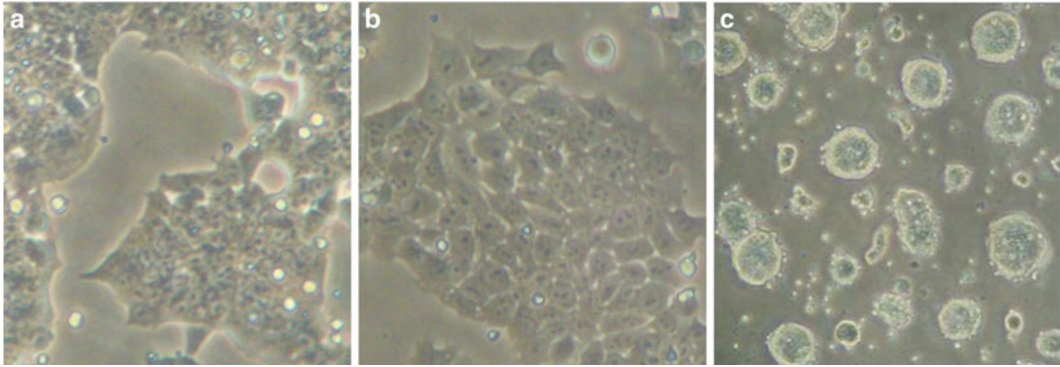

Fig. 1 Morphology of ESCs under the phase contrast microscope. (**a**) ESCs in serum/LIF condition. (**b**) ESCs in CDM/2i/LIF condition on plates coated with laminin (spread and attached colonies). (**c**) ESCs in CDM/2i/LIF condition on plates coated with gelatin (ball-like colonies)

immunofluorescence, for example. On the other hand, Laminin coating leads to full attachment and spreading of these cells, which is convenient for further manipulations (Fig. 1).

5. The aliquot of Laminin should be thawed gently at +4 °C and then diluted directly in DPBS in the dish that needs to be coated. It is not possible to use two times the same diluted Laminin to make the coating of a second dish, as not enough Laminin remains after the first incubation.
6. ESCs in serum condition can also be cultured on a layer of feeder cells. In this case, it is necessary to get rid of feeder cells in order to convert ESC into the ground state. To accomplish this, the pre-plating step is essential: after passaging the ESC with feeders, cells are plated twice on noncoated cell culture plates for 20 min before the final plating on laminin-coated dishes. Because ESCs need many hours to attach, during the pre-plating steps, only the feeder cells will have time to attach.
7. When performing the switching, so starting from ESCs in serum (with or without feeders), it is necessary to perform the washing with DPBS and the tryspin treatment should be shorter about 2 min. Otherwise, during normal passage of ESCs that are already in 2i medium, no washing step is required.
8. ESCs in 2i condition are usually passaged every 3 or 4 days with a dilution 1/6 or 1/8 and the medium is changed every 2 days.
9. In first two or three passages after the switching of ESCs from serum/LIF condition to 2i/LIF condition, cells usually appear not completely attached to dished showing some flat colonies and some ball-like colonies typical of standard culture of ESCs in N2B27/2i/LIF on gelatin-coated dishes

(*see* Fig. 1). After 2 weeks of culture of ESCs under 2i/LIF condition, cells are fully attached and we could consider that they have reached the "ground state" (11).

10. Also after freezing and thawing, ESCs in 2i may not be completely attached and spread during the first passage.

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Generation of Embryonic Stem Cells in Rabbits

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Abstract

Here we have described a procedure to generate embryonic stem cell (ESC) lines from rabbit preimplantation blastocysts. We have also provided detailed procedures to characterize the resulting ESC lines, such as the analysis of pluripotency marker expression by reverse transcription quantitative polymerase chain reaction, immunolabeling, and fluorescence-associated cell sorting; evaluation of pluripotency by teratoma production; and assessment of genetic stability by karyotyping.

Keywords: Embryonic stem cells, Derivation, Rabbit, Pluripotency, Preimplantation embryos

1 Introduction

Pluripotency defines the capacity of a single cell to differentiate into all cell types, including germ cells, in a developing fetus. In mice, pluripotency can be captured and propagated in vitro in the form of embryonic stem cells (ESCs) that are derived from the early epiblasts of blastocysts (1, 2). Mouse ESCs can colonize a blastocyst and can contribute to the development of all tissue types, including germ cells, after embryo transfer into surrogate mothers. This has led to the generation of mutant mice harboring inactivated genes and subsequently to numerous breakthroughs in the elucidation of genetic pathways that regulate embryonic and fetal development in rodents (3). Development of similar technologies in rabbits offers a potentially broad scope of applications, including generation of models to study early development in humans and human diseases, and generation of bioreactors (4). Here we have described a procedure to generate ESC lines from rabbit preimplantation blastocysts. A thorough molecular and functional analysis of these lines is described in Osteil et al. (5).

*These authors contributed equally to this work

2 Materials

2.1 Ovarian Stimulation

1. 50 μg/mL porcine follicle-stimulating hormone (pFSH; Stimufol®, Reprobiol): To prepare 9 μg/mL working solution, dilute 0.9 mL Stimufol with 4.1 mL phosphate-buffered saline (PBS) without Mg^{2+}/Ca^{2+} (14190–169; Life Technologies)
2. 0.4 μg/mL buserelin acetate (Receptal®, Intervet)
3. 1-mL syringe and 200-μL tips
4. Rabbit sperm: sperm was freshly collected using an artificial vagina (COLLAP, 022931, IMV Technologies) and diluted up to 10× with GALAP solution (002269, IMV Technologies)

2.2 Embryo Collection and Culture

1. Sterilized small scissor, forceps, and scalpels (sterilize in a dry heat oven for 2 h at 180 °C)
2. 20-mL syringe, 200-μL tips, aspirator tube assembly for calibrated microcapillary pipettes (A5177, Sigma-Aldrich), 100-mm petri dishes, and 4-well cell culture plates (144444; Nunc)
3. Glass capillaries of 1.5-/1.17-mm diameter (GC150T10, Phymep): Pull the capillaries to obtain different diameters at one extremity, and sterilize them in dry heat oven for 2 h at 180 °C
4. Binocular stereoscopic microscope and incubator maintained at 38 °C and 5 % CO_2
5. Flushing medium: PBS without Mg^{2+}/Ca^{2+} (*see* Section 2.1, **item 1**) supplemented with 10 % fetal bovine serum (FBS; Hyclone, SH30073003, Fisher Scientific)
6. RDH medium: For 100 mL, mix 33.33 mL DMEM-GlutaMAX® (31966, Life Technologies), 33.33 mL RPMI-GlutaMAX® (61870, Life Technologies), and 33.33 mL Ham's F10-GlutaMAX® (41550, Life Technologies); filter the solution through a 0.22-μM polyethersulfone (PES) syringe filter (SLGP033RS, Merck-Millipore) and store at −20 °C. Before use, add 0.3 g bovine serum albumin (A3311, Sigma-Aldrich) suitable for mouse embryo cell culture and 0.0625 g taurine (T8691, Sigma-Aldrich) in a 100 mL solution. Adjust the pH at 7.6 and filter the solution through a 0.22-μM PES syringe filter. The solution can be stored at 4 °C for a maximum of 1 week

2.3 Feeder Cell Preparation

1. 4-well Nunc culture plates (*see* Section 2.2, **item 2**), 35-mm non-treated Corning cell culture dishes (430-588, Corning), 15-mL and 50-mL Falcon tubes
2. Low-speed centrifuge and incubator maintained at 38 °C and 5 % CO_2
3. PBS (*see* Section 2.1, **item 1**) and FBS (*see* Section 2.2, **item 5**)

4. Fibroblast culture medium: To 500 mL of DMEM (DMEM with high glucose, L-glutamine, phenol red, and sodium pyruvate; 41966-052, Life Technologies), add 55 mL of FBS (10 %), 5.5 mL of 100× penicillin/streptomycin/glutamine (50 mg/mL PSG; 10378-016, Life Technologies), 5.5 mL of 100× nonessential amino acids (NEAA; 11140-035, Life Technologies), and 1.1 mL of 50 mM β-mercaptoethanol (31350-010, Life Technologies)
5. 0.1 % gelatin: For 100 mL, dilute 5 mL of 2 % gelatin (gelatin solution type B; G1393 Sigma-Aldrich) with 95 mL PBS. Sterilize in steam autoclave for 20 min at 120 °C
6. 1× trypsin: dilute 1 mL 10× trypsin (0.5 % trypsin–EDTA w/o phenol red; 15400-054, Life Technologies) with 9 mL PBS
7. 5 μg/mL mitomycin-C (M4287, Sigma-Aldrich): Dissolve one vial containing 2 mg of mitomycin-C in DMEM to prepare a 10× stock solution of 50 μg/mL concentration. Store aliquots of 1 mL at −20 °C. Before use, dilute one aliquot containing 1 mL of 10× mitomycin-C with 9 mL of DMEM or fibroblast medium to a final concentration of 5 μg/mL (1×)
8. Mouse Embryonic Fibroblasts (MEF) prepared from 12.5-day-old embryos collected from the OF1 strain (Charles River) (*see* **Note 1**)

2.4 Embryonic Stem Cell Derivation

1. Aspirator tube assembly, glass capillaries, 100-mm petri dishes, and 4-well cell culture plates (*see* Section 2.2, **items 2** and **3**)
2. Binocular stereoscopic microscope; inverted microscope; incubator maintained at 38 °C and 5 % CO_2; and tri-gas incubator maintained at 38 °C, 5 % CO_2, and 5 % O_2
3. RDH medium (*see* Section 2.2, **item 6**)
4. 199 medium with HEPES (12340-030, Life Technologies)
5. 5 mg/mL protease E (P5147, Sigma-Aldrich): Dilute 100 mg of protease E dry powder in 20 mL of embryo-holding medium (019449, IMV Technologies), filter the solution through a 0.22-μM PES syringe filter, aliquot, and store at −20 °C
6. Human recombinant basic fibroblast growth factor (FGF2; 01-106, Merck-Millipore): Prepare 100 ng/μL stock solution by dissolving one vial (25 μg) of FGF2 in 250 μL PBS. Prepare 10-μL aliquots and store at −20 °C. Prepare 10 ng/μL working solution by diluting the stock solution with PBS. The working solution can be stored at 4 °C for a maximum of 1 week. Immediately before use, add the desired volume of FGF2 working solution to warm rabbit ESC (rbESC) culture medium to achieve a final concentration of 13 ng/mL
7. PSG, NEAA, and β-mercaptoethanol (*see* Section 2.3, **item 4**)

8. Knockout serum replacement (KOSR; 10828-028, Life Technologies)
9. 100 mM sodium pyruvate (11360-039, Life Technologies); working concentration of 1 mM
10. rbESC culture medium: To 500 mL DMEM/F12 (DMEM/F12 with phenol red and without L-glutamine; 21331-020, Life Technologies), add 130 mL of 20 % KOSR, 6.5 mL of 100× PSG, 6.5 mL of 100× NEAA, 6.5 mL of 100 mM sodium pyruvate, and 1.3 mL of 50 mM β-mercaptoethanol. Next add FGF2 extemporaneously to achieve a final concentration of 13 ng/mL

2.5 Embryonic Stem Cell Culture

1. Aspirator tube assembly and pulled and sterilized glass capillaries (*see* Section 2.2, **items 2** and **3**)
2. 35-mm Corning culture dishes (*see* Section 2.3, **item 1**)
3. Inverted microscope and tri-gas incubator maintained at 38 °C, 5 % CO_2, and 5 % O_2
4. 1 mg/mL collagenase II (C6885, Sigma-Aldrich): Dilute 100 mg of collagenase II dry powder in 100 mL PBS, filter the solution through a 0.22-μM PES syringe filter, aliquot, and store at −20 °C
5. rbESC culture medium (*see* Section 2.4, **item 10**)

2.6 Analysis of Pluripotency Markers by Immunofluorescent Microscopy and Flow Cytometry Assays

1. Glass coverslips (140540, Dutsher), glass slides (100258, Dutscher), 24-well culture plates, tubes for flow cytometry
2. 0.1 % gelatin, FBS, PBS, 1× trypsin (*see* Section 2.3, **items 5** and **6**)
3. 37 % aqueous solution of paraformaldehyde (PFA; 15714, Delta Microscopy Sciences): For a 4 % working solution, dilute 5.4 mL of 37 % PFA with 44.6 mL PBS
4. 30 % solution of bovine serum albumin (BSA) in PBS (A9576, Sigma-Aldrich)
5. 10× tris buffered salt (TBS): 200 mM Tris–HCl (pH 7.4), 0.9 % NaCl (T5912, Sigma-Aldrich); dilute tenfold with water to obtain a working solution
6. TBS–Triton: 0.4 % Triton X-100 (T8787, Sigma-Aldrich) in TBS. For a 1× solution, dilute 0.4 mL Triton X-100 with 100 mL TBS
7. TBS–Tween: For preparing 0.1 % Tween 20 (P5927, Sigma-Aldrich) in TBS, dilute 1 mL of Tween 20 with 1 L TBS
8. Blocking solution: For preparing TBS containing 10 % FBS and 0.1 % BSA, dilute 10 mL of FBS with 90 mL of TBS and add 0.34 mL of 30 % BSA

Table 1
List of antibodies

Molecule	Antibodies	Dilution	Manufacturer	Reference	Method
OCT-4	1°: Anti-Oct-4 Rabbit IgG 2°: AF555[a] Goat Anti-Rabbit IgG	1/300 1/1,000	Santa Cruz Invitrogen	SC-9081 A21429	IF[b]
SSEA1	PE[c] Anti-SSEA1 Mouse IgM AF647[d] Anti-SSEA1 Mouse IgM	1/50 1/100	R&D Systems Santa Cruz	FAB2155P SC-21702	IF FC[e]
SSEA4	1°: Anti-SSEA4 Mouse IgG 2°: AF555 Goat Anti-Mouse IgG 1°: Anti-SSEA4 Mouse IgG 2°: AF488[f] Goat anti-Mouse IgG	1/100 1/100 1/200 1/1,000	Millipore Invitrogen Santa Cruz Invitrogen	MAB4304 A21422 SC-21704 A11017	IF FC
Tra-1-60	1°: Anti-Tra-1-60 Mouse IgM 2°: AF555 Goat Anti-Mouse IgM	1/50 1/500	Millipore Invitrogen	MAB4360 A21426	IF
E-CADH	PE Anti-E-Cadherin Rat IgG2A	1/50	R&D Systems	FAB7481P	IF
N-CADH	1°: Anti-N-Cadherin Rabbit IgG 2°: Rhod[g] Goat anti-Rabbit IgG	1/200 1/1,000	Santa Cruz Molecular Probes	SC-7939 RG314	IF
CD90	1°: Anti-CD90 Mouse IgG 2°: AF555 Goat Anti-Mouse IgM	1/50 1/500	Pharmingen™ Invitrogen	550402 A21426	IF

Fluorochromes: [a]Alexa Fluor 555; [c]Phycoerythrin; [d]Alexa Fluor 647; [f]Alexa Fluor 488, [g]Rhodamine
Methods: [b]Immunostaining; [e]Flow cytometry

9. Hoechst 33342: Dissolve 25 mg of bis-benzimide H 33342 trihydrochloride (B2261, Sigma-Aldrich) in 25 mL water to prepare aliquots of 1 mg/mL and store at −20 °C. Dilute 1 μL of the stock solution with 1 mL PBS to obtain a 1 μg/mL working solution
10. Antibodies for Oct4, SSEA-1, SSEA-4, TRA-1-60, E-cadherin, N-cadherin, and CD90: *see* Table 1
11. Mounting medium (M1289, Sigma-Aldrich)
12. DPX medium (SEA-1304-00A, CellPath)

2.7 Analysis of Pluripotency Markers by RT-qPCR

1. Reagents for mRNA extraction: RNeasy mini kit (74106, Qiagen) and RNase-free DNAse Set (79254, Qiagen)
2. Reagent for reverse transcription of mRNA into cDNA: RNA to cDNA kit (4387406, Life Technologies)
3. Reagent for real-time PCR: Fast SYBR Green Master MIX (4385618, Life Technologies)
4. StepOnePlus real-time PCR system (Applied Biosystems) and low speed centrifuge
5. 96-well PCR microplates (4346906, Life Technologies) and Optical adhesive covers (4311971, Life Technologies)
6. Primers (100 μM) specific for pluripotency genes (*see* Table 2). Prepare a working solution of primer pairs (each 10 μM) by

Table 2
List of primers used for RT-qPCR analysis

Gene	Forward primer	Reverse primer
Tbp	CTTGGCTCCTGTGCACACCATT	ATCCCAAGCGGTTTGCTGCTGT
Gadph	TTCCACGGCACGGTCAAGGC	GGGCACCAGCATCACCCCAC
Nanog	CACTGATGCCCGTGGTGCCC	AGCGGAGAGGCGGTGTCTGT
Oct4	CCTGCTCTGGGCTCCCCCAT	TGACCTCTGCCTCCACCCCG
Klf4	TCCGGCAGGTGCCCCGAATA	CTCCGCCGCTCTCCAGGTCT
Dazl	CACAGTGGCCTACTGGGGAACA	TTCGGCGCCTGGGTCAACTT
Tbx3	TGGATTCGGGCTCGGAACTGA	AGCCGCTGGATGCTCTGAAGT
Blimp1	AGCGGCGAACGGCCTTTCAAAT	GACCTGGCATTCATGCGGCTTT
Lefty2	ACTGCCGCATTGCCCATGAT	AGCTGCACTGCTTCACCCTCAT
Dax1	GCCTGCAGTGCGTGAAGTACA	CGGTGCGTCATCCTGACGTG
Fbxo15	AGGCTCGGCCACTGTTCCTT	CACGTGGAGCTGGTAGCCATGT
Pecam1	AGAGGAGCTGGAGCAGGTGTTAAT	GCTGATGTGGAACTTCGGAACAGA
Piwil2	TGACCTTTCCGGATCCTTCAGTGT	TCCGAACTCCCTCTTCCAAGCATT
Gbx2	AACGCGTGAAGGCGGGCAAT	TGCTGGTGCTGGCTCCGAAT
Rex1	AGCCCAGCAGGCAGAAATGGAA	TGGTCAGTCTCACAGGGCACAT
Fgf4	ACGCAGACACGAGCGACAGC	CGGCTGGCCACGCCAAAGAT
Otx2	TCCGGCTCGGGAAGTGAGTT	GGAGCACTGCTGCTCGCAAT
Cldn6	GCAGCCTCGGGCCTTTTGTTG	TCGGGCCAGACGCTGAGTAG
Pitx2	ACTAGCGCGCAGCTCAAGGA	CAGCTCCTCGCGCGTGTAAA
Cdx2	CTCAACCTGGCGCCGCAGAA	GCGCGCTGTCCAAGTTCGC
Cdh1	TGCACAGGCCGGAAACCAGT	ACGGCCTTCAGCGTGACCTT
Cdh2	CCGTGGCAGCTGGACTGGAT	GATGACGGCCGTGGCTGTGT
Esrrb	CGTGGAGGCCGCCAGAAGTA	TCTGGCTCGGCCACCAAGAG
Tcfcp2l1	AGAAAAGGGCGTGCCGTTCC	TGGCAGCTGGCTGAGTGCAA

mixing 10 μL of each reverse and forward primer with 80 μL water. Primers are chosen based on the known mouse pluripotency genes (6, 7) and rabbit homologs annotated in sequence databases (*Oct4*, *Nanog*, *Klf4*, *Dazl*, *Tbx3*, *Blimp1*, *Lefty2*, *Dax1*, *Fbxo15*, *Pecam1*, *Piwil2*, *Gbx2*, *Rex1*, *Fgf4*, *Otx2*, *Clnd6*, *Pitx2*, *Cdx2*, *Cdh1*, *Cdh2*, *Esrrb*, *Tcfcp2l1*). To avoid the amplification of mouse genes originating from contaminating feeder cells, these primers must be designed to specifically amplify the rabbit genes

2.8 Teratoma Formation

1. Eight to ten-week-old immunodeficient mouse strain, Fox Chase SCID (CB17/lcr-Prdcscid/lcrlcoCrl; strain 236, Charles River): two mice per tested RbESC line
2. Glass capillaries (*see* Section 2.2, **item 3**) and glass slides (*see* Section 2.6, **item 1**)
3. Sterilized small scissor, scalpels, screed clips, and forceps (dry-heat oven, 2 h, 180 °C)
4. Anesthesia solution: Mix ¼ volume of 2 % Rompun solution (Bayer Healthcare) with ¾ volume of Imalgene 1000 (Merial)
5. 4 % PFA (*see* Section 2.6, **item 3**) and PBS (*see* Section 2.1, **item 1**)
6. Tissue-Tek™ CRYO-OCT Compound (OCT; 14-373-65, Fisher Scientific)
7. Hematoxylin–Phloxine–Saffron Stain (HPS; k023, Poly Scientific R&D Corp)

2.9 Karyotyping

1. 175-cm^2 Nunc culture flask (055421, Dutscher)
2. KaryoMAX Colcemid solution: 10-μg/mL solution of N-desacetyl-N-methylocolchicine in PBS (15212-012, Life Technologies). For preparing a 0.4-μg/mL working solution, dilute 2 mL of karyoMAX with 48 mL of RbESC culture medium
3. Shock solution: For preparing 75 mM KCl (P9333, Sigma-Aldrich), dissolve 0.28 g KCL in 50 mL water
4. Fixation solution: Mix one volume of glacial acetic acid (1005706, Sigma-Aldrich) with three volumes of ethanol (02860, Sigma-Aldrich) and store at −20 °C
5. Digestion solution: 0.045 % trypsin in Ca++- and Mg++-free Tyrode Ringer's saline. Prepare Ringer's buffer by dissolving 8 g NaCl (S7653, Sigma-Aldrich), 0.3 g KCl (P9333, Sigma-Aldrich), 0.093 g $NaH_2PO_4 \cdot 5H_2O$ (71505, Sigma-Aldrich), 0.025 g KH_2PO_4 (P5655, Sigma-Aldrich), 1 g NaHCO3 (S5761, Sigma-Aldrich), and 2 g glucose (G0350500, Sigma-Aldrich) in 1 L water and adjust the pH to 7.6–7.7. Dilute 1.8 mL of 2.5 % trypsin solution (59427C, Sigma-Aldrich) with 100 mL of ringer's buffer to obtain 0.045 % trypsin.
6. Staining solution: Mix 90 mL water, 3 mL of KaryoMAX Giemsa (10092-054, Life Technologies), 3 mL methanol (322415, Sigma-Aldrich), and 3 mL of 0.1 M citric acid (5.35 g in 250 mL water) (251275, Sigma-Aldrich) in order. Verify that the pH of the solution is between 2 and 3, and adjust the pH to 6.8 with 0.5 M Na_2HPO_4 (17.9 g in 250 mL water) (255793, Sigma-Aldrich).
7. Glass slides (*see* Section 2.6, **item 1**): Degrease the glass slides with soap solution, rinse with water, and store immersed in 33 % ethanol (33 mL ethanol and 67 mL water) at 4 °C

2.10 Freezing and Thawing MEFs and rbESCs

1. Freezing solution for MEFs: A mixture of 80 % FBS (*see* Section 2.2, **item 5**) and 20 % DMSO (D2650, Sigma-Aldrich).
2. Freezing solution for rbESCs: A mixture of 80 % KOSR (*see* Section 2.4, **item 8**) and 20 % DMSO.
3. 1.8-mL Nunc Cryotubes (055003, Dutscher).
4. Nalgene cell freezing container (C1562, Sigma-Aldrich).
5. Isopropanol (I9516, Sigma-Aldrich).

3 Methods

Perform all the procedures in Class II Biological Safety Cabinet at room temperature unless otherwise specified. It is recommended to place the microscope under the cabinet for manual passaging of cells. Embryo manipulations and dissections should be performed using glass capillaries of different diameters attached to an aspirator tube holder (commonly called "mouth pipette").

3.1 Rabbit Ovarian Stimulation

1. Stimulate the ovaries by subcutaneously injecting 9 μg/mL of pFSH solution at 12-h intervals for 3 days as follows: inject 0.5 mL pFSH twice on day 1 (morning and evening), 1 mL pFSH twice on day 2 (morning and evening), and 0.5 mL pFSH in the morning of day 3 (Fig. 1).
2. Collect rabbit sperm by using an artificial vagina heated to 42 °C. Dilute the sperm to 5–10× concentration with GALAP solution and store at 4 °C for 2 days maximum.
3. Induce ovulation on day 3 at 12 h after the last injection of pFSH by injecting 0.4 mL Receptal (1.6 μg buserelin acetate) intramuscularly.
4. Perform artificial insemination immediately by injecting 0.5 mL of the diluted sperm into the vagina by using a syringe with a tip.

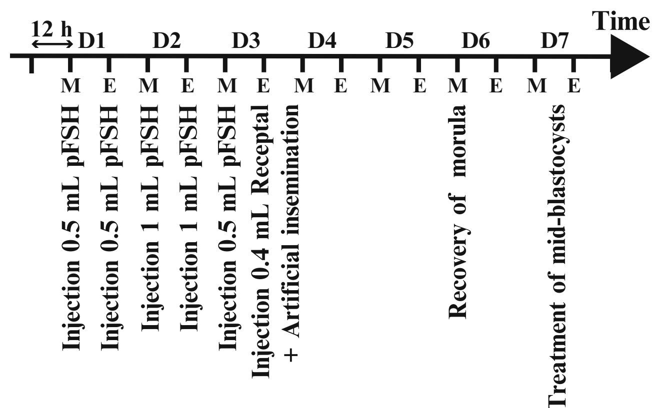

Fig. 1 Time schedule of ovarian stimulation and rabbit embryo production

3.2 Embryo Collection and Culture

1. To collect embryos at the morula stage, sacrifice the females 60 h after the insemination.
2. Open the peritoneal cavity and remove the entire genital tract by using a scalpel.
3. Dissect the two uterine horns to eliminate fat, and dissociate them.
4. Hold the genital tract between two fingers in order to release the oviduct entry and place the uterine horn in a petri dish.
5. Perfuse each oviduct with 10–20 mL of flushing medium by introducing the tip of the syringe into the oviduct entry.
6. Identify embryos at the morula stage with a binocular stereoscopic microscope, and transfer the embryos by using a glass capillary into a well of 4-well cell culture plate containing 500 μL RDH medium.
7. Wash the embryos twice with the RDH medium and transfer them into another well containing fresh medium. Incubate the embryos at 38 °C in 5 % CO2 for 12–24 h, until they reach the blastocyst stage.

3.3 Feeder Cell Preparation

1. Day 1: Thaw a vial of OF1 MEFs (*see* Section 3.10) and plate the cells in three 100-mm culture dishes, each containing 10 mL of fresh culture medium. Incubate the cells at 37 °C in 5 % CO_2 for 72 h.
2. Day 4: Replace the culture medium over MEFs with 5 mL of 1× mitomycin-C. Incubate the cells at 37 °C in 5 % CO_2 for 2–3 h. Remove the mitomycin-C and rinse the cells five times with 5 mL of PBS. Then, to each dish, add 1 mL of 1× trypsin and incubate the dishes for 5 min at 37 °C. After incubation, add 1 mL of fibroblast medium to each dish to stop the enzymatic reaction. Dislodge and dissociate the cells by repeated pipetting. Transfer the cell suspension into a 15-mL Falcon tube containing 10 mL of fibroblast medium, centrifuge for 5 min at 300 g, and resuspend the cell pellet in 10 mL of fresh medium. Count the cells using a Malassez counting chamber. Incubate culture dishes with 0,1 % gelatin for 30 min. Plate the cells onto gelatin-coated dishes at a density of 1.2×10^4 cells/cm^2 (1.2×10^5 cells in 2 mL medium for one 35-mm culture dish, 3.1×10^4 cells in 1 mL medium for 1 well of a 24-well plate, 2.3×10^4 cells in 1 mL medium for 1 well of a 4-well plate). Incubate the cells overnight at 37 °C in 5 % CO_2 before use. Inactivated MEFs must be used within 3 days.

3.4 Embryonic Stem Cell Derivation

1. Prepare a dish lid with drops of embryo-holding medium (three drops), protease E (one central drop), and 199 medium (four drops) as described in Fig. 2a.

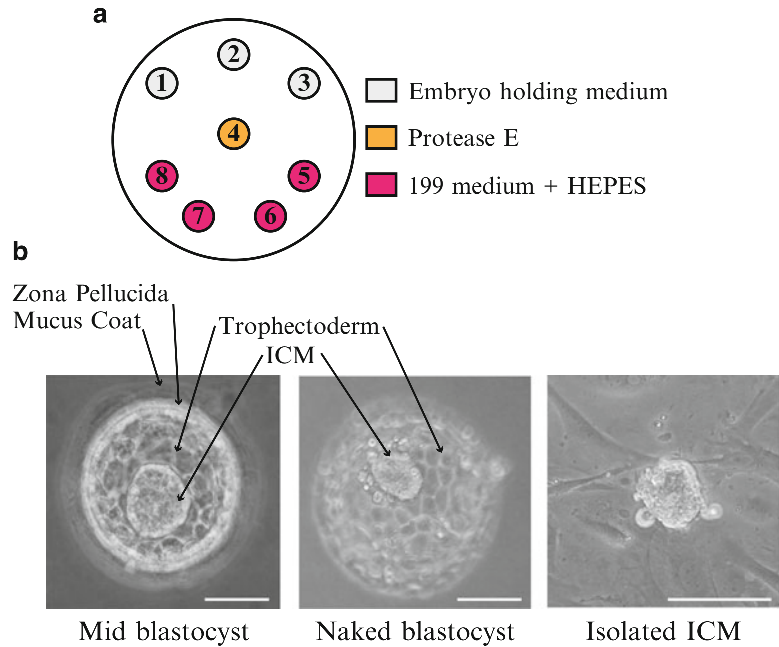

Fig. 2 Rabbit embryo treatment. (**a**) Treatment steps. (**b**) Effect of enzymatic treatment on mid-blastocyst stage. Scale bar = 50 μm

2. Use mid-blastocysts with well-defined inner cell mass (ICM; Fig. 2b). Do not treat >20 blastocysts at the same time. Rinse the blastocysts successively using three drops of embryo-holding medium. Next, place the rinsed blastocysts for 5–7 min in the protease drop to digest the mucus coat. At the end of treatment, this mucus coat will appear relaxed, indicating that it is ready to be removed.
3. Next, rinse the blastocysts in the first two drops of 199 medium to remove protease E and incubate them further in the third drop.
4. Take a glass capillary having an opening diameter slightly larger than the blastocyst. Pipette 199 medium up and down twice to coat the inner side of the glass capillary. Then, aspirate the embryo and gently pipette it up and down to remove the mucus coat.
5. Take a glass capillary having an opening diameter slightly smaller than the blastocyst. Pipette the embryo up and down to remove the zona pellucida. Transfer the "naked" embryo into the last drop of the 199 medium (Fig. 2b).
6. After all the embryos have been treated, transfer each embryo into 1 well of a 4-well cell culture plate coated with feeder cells and containing rbESC culture medium. Incubate the embryos

overnight at 38 °C in 5 % CO_2 and 5 % O_2, to allow them to recover.

7. Take a glass capillary with an opening diameter slightly smaller than the embryo and pipette the embryos up and down to cut them in two pieces. Discard the piece of trophectoderm that does not contain the ICM. Let the second piece plate overnight.
8. Discard the medium, and replace it with fresh rbESC culture medium every day thereafter.
9. After 5–7 days, the ICM develops into an outgrowth covered by differentiated cells that form a veil. Remove the veil with a sterile glass capillary attached to a mouth pipette by gentle suction and tearing. If the outgrowth is well developed, it remains attached to the feeder layer (Fig. 3).
10. Let the outgrowth develop for an additional day. A very large and flat colony is now ready for passaging (Fig. 3). By using a

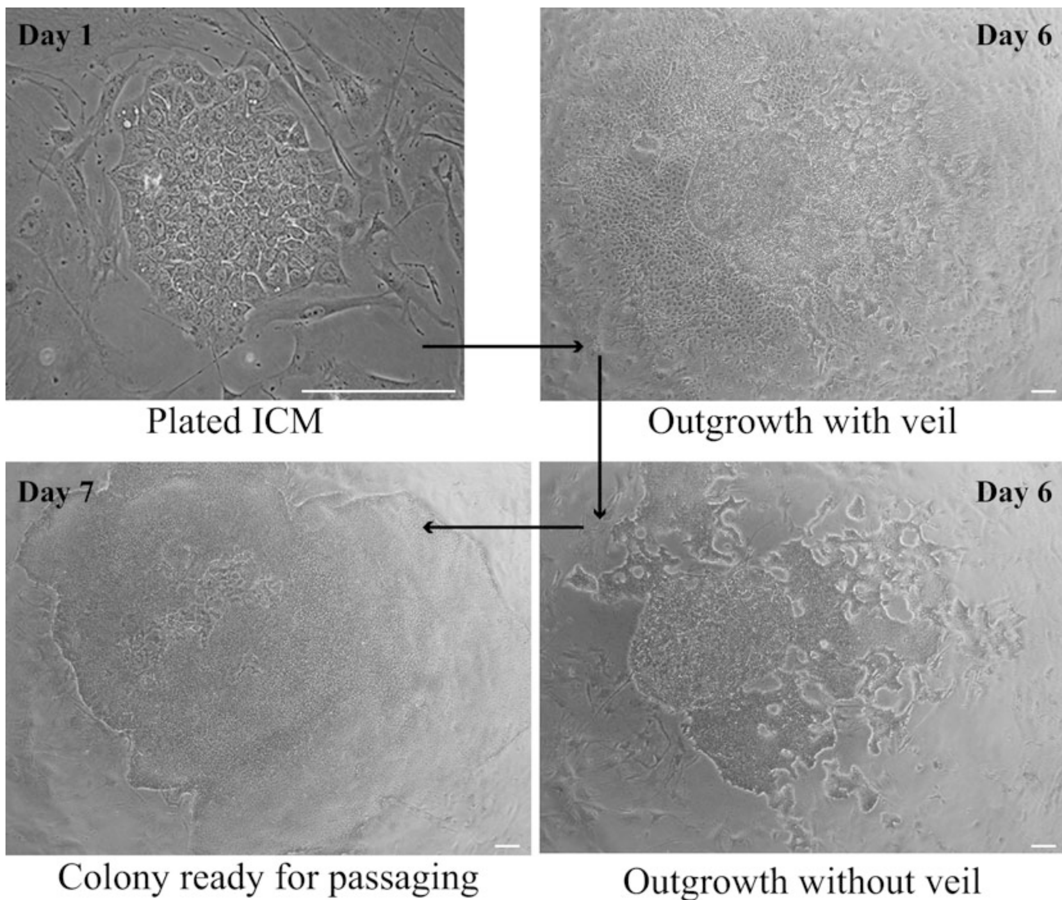

Fig. 3 Development of isolated ICM in culture. Appearance of outgrowth before and after the elimination of the veil formed by the differentiated cells. Scale bar = 50 μm

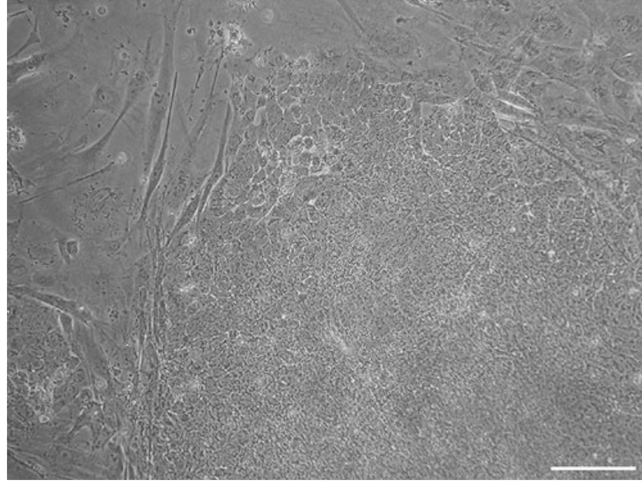

Fig. 4 Morphology of rbESCs. Phase-contrast image of an rbESC line at passage 28. Elongated feeder cells surround flat colonies of highly packed rbESCs. Scale bar = 50 μm

sterile glass capillary cut the colony into small clumps containing 20–30 cells under a microscope. Next, transfer these clumps of cells into a 35-mm gelatin-coated dish containing fresh feeder cells, previously conditioned with the rbESC medium.

3.5 Embryonic Stem Cell Culture

1. Change the rbESC culture medium every day to avoid spontaneous differentiation. RbESCs grow as flat colonies of tightly packed cells (Fig. 4).
2. Proceed to cell passaging every 3–4 days depending on colony density and size. Treat cells for 1 min with 1 mL collagenase II at 1 mg/mL that was pre-warmed at 37 °C. Gently rinse the cells with 1 mL PBS. Add 2 mL of the fresh culture medium without FGF2. Divide the colonies into several clumps under the microscope and transfer them into a new culture dish coated with feeders and conditioned with a medium supplemented with FGF2.
3. Colonies from one 35-mm culture dish should be passaged routinely in two to three 35-mm culture dishes.
4. RbESC lines are established between the sixth and the tenth passages, when cells can be amplified without extensive differentiation.

3.6 Analysis of Pluripotency Markers by Immunofluorescent Microscopy and Flow Cytometry Assays

1. For in situ immunolabeling, prepare glass coverslips by incubating with 0.1 % gelatin for 10 min, and then with FBS overnight. Seed the coverslips with feeder cells as described earlier (*see* Section 3.3, **step 2**). Coverslips can be placed at the bottom of 24-well plates.

2. Plate 1×10^5 rbESCs onto feeder cells maintained on glass coverslips. Incubate the cells at 38 °C, in 5 % CO_2 and 5 % O_2 for 48 h.
3. Rinse the cells once with PBS, fix by incubating for 20 min with 2 % PFA (first add 250 μL PBS per well, then add 250 μL 4 % PFA per well), and rinse twice with PBS. Fixed cells can be store at 4 °C.
4. For the analysis of intracellular antigens, permeabilize the cells with TBS–Triton (500 μL/well) for 20 min and wash three times (10 min each) with TBS.
5. Block nonspecific binding sites with blocking solution (500 μL/well) for 1 h and incubate overnight at 4 °C with primary antibodies (Oct4, SSEA-1, SSEA-4, TRA-1-60, E-cadherin, N-cadherin, and CD90) diluted in blocking solution (Table 1).
6. Wash the cells with TBS–Tween three times for 10 min each and incubate the cells with fluorochrome-conjugated secondary antibodies (Table 1) at room temperature for 1 h.
7. Rinse the cells with TBS–Tween three times for 10 min each and stain the nuclei by incubating the samples for 5 min with a 1-μg/ml solution of Hoechst 33342.
8. Rinse the cells four times with TBS (5 min each). Place the coverslips on glass slides with 7 μL of mounting medium and fix with DPX medium. Examine the cells under a conventional fluorescence microscope fitted with appropriate filters for Hoescht (blue), and Phycoerythrin, Rhodamine, or Alexa Fluor 555 (red) (Fig. 5a).
9. For flow cytometry, dissociate the cells using 1× trypsin and label the cells in suspension using the protocol and the antibodies (SSEA-1, SSEA-4) described above (Fig. 5b).
10. Analyze the labeled cells using a flow cytometer with the help of red (633 nm) and blue (488 nm) lasers for detection of Alexa Fluor 647 or 488 labeling, respectively.

3.7 Analysis of Pluripotency Markers by RT-qPCR

1. Extract mRNA from dry cell pellets using the RNeasy mini kit and treat with DNAse I (follow the manufacturer's protocol). Measure the concentration of RNA in solution with a NanoDrop spectrophotometer. Dilute the RNA to a concentration of 50 ng/μL using RNAse-free water. Keep the RNA solution on ice or store at −80 °C.
2. Perform reverse transcription of the RNA using the RNA to cDNA kit. Mix 10 μL of RT buffer containing the dNTPs and the random primers with 9 μL of diluted RNA (450 ng) and 1 μL reverse transcriptase. Incubate for 5 min at 70 °C and then

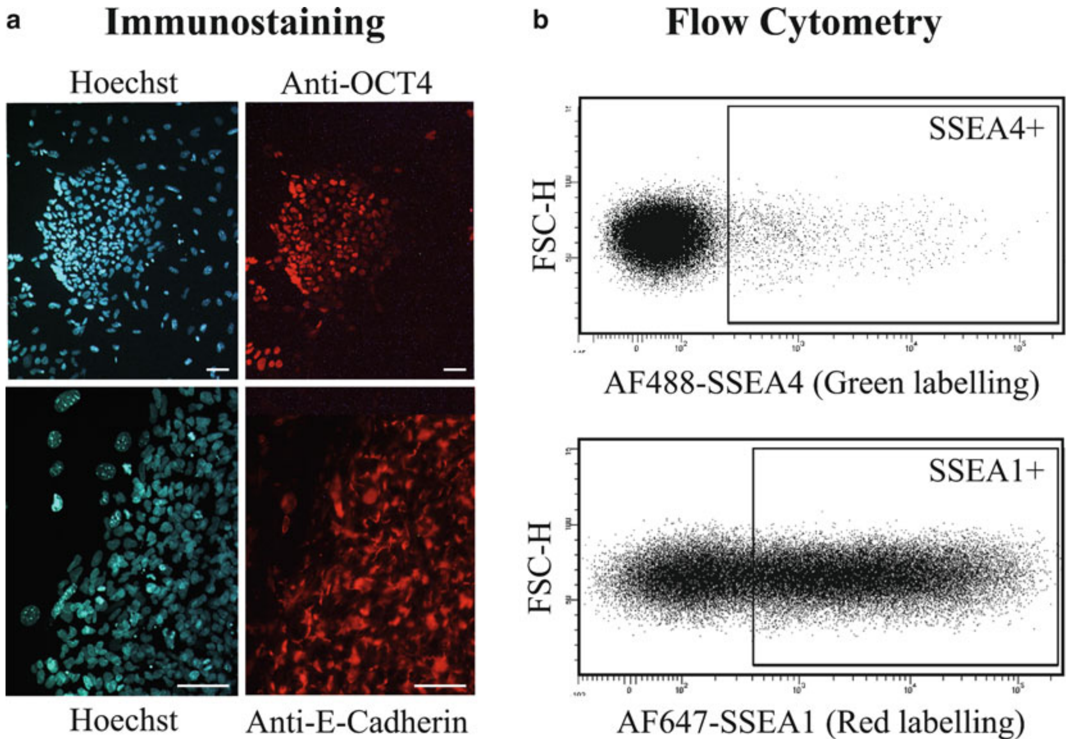

Fig. 5 Detection of pluripotency markers with specific antibodies. (**a**) Immunofluorescence detection of OCT4 and E-Cadherin in rbESCs after staining with specific antibodies, and labeling of nuclei with bis-benzimide (Hoechst). Scale bar = 50 μm. (**b**) Flow cytometry analysis of rbESCs showing the heterogeneity in the expression of SSEA1 and SSEA4 pluripotency markers

for 45 min at 37 °C. Dilute the cDNA solution fivefold with water and store at −20 °C.

3. Prepare PCR reaction mixtures in microplates. For this purpose, mix 5 μL of SYBR® Green Mix, 0.44 μL of primer mix, and 3.6 μL water. Add 9 μL of the mixture to 1 well of a 96-well microplate. To this, add 1 μL of diluted cDNA. Perform each PCR reaction in triplicate. For each primer pair used, run a negative control in triplicate, where the reaction mixture is prepared by substituting cDNA with water. Close the microplate with an optical adhesive cover and centrifuge the plate for 1 min at 800 g.

4. Run the qPCR reaction using the StepOnePlus real-time PCR system according to the manufacturer's instructions. Use 40 amplification cycles and an annealing temperature of 60 °C for the reaction. At the end of the amplification, analyze the melt-curve and verify that only the desired PCR products are formed. Using the StepOnePlus Software V2.1 (Applied Biosystems), determine the amplification efficiency for both target

and reference genes from the relative values of the calibrator-normalized target gene expression. Then, normalize the expression of the target genes to those of the rabbit *Gadph* and *Tbp* (TATA-box binding protein).

3.8 Teratoma Formation

1. All material and food used for breeding the mice should be sterilized to avoid infection.
2. Teratoma formation can be induced by injecting rbESCs under the kidney capsule of the Severely Compromised ImmunoDeficient (SCID) mice. Inject rbESCs into one kidney of each mouse. Each rbESC line must be injected into at least two mice.
3. Preparation of rbESCs: Dissociate the rbESCs using 1× trypsin, count the cells, and centrifuge the cell suspension. Resuspend 2.5×10^6 rbESCs in 10 μL PBS.
4. Preparation of the mouse: Anesthetize the mouse by intramuscular injection of 1.4 μL/g of anesthesia mix. Allow the mouse to rest on its stomach and make a small incision (approximately 1 cm) in the skin and muscle of the back (parallel to the spinal column and at the same level as the top of the hip). Part the skin and muscle to see the kidney.
5. Inject 10 μL of the rbESC suspension under the kidney capsule using a glass capillary and suture the wound with screed clips. Allow the mouse to recover.
6. For the next 4–8 weeks, monitor the tumor growth by following the regular palpation of the back of the mouse. Sacrifice the mice by cervical dislocation when the manipulated kidney is approximately twice as big as the normal kidney. Surgically remove the damaged kidney.
7. Fix the kidney along with the tumor for 1 week with 4 % PFA at 4 °C.
8. Rinse the organ with PBS, embed in OCT compound, and prepare cryosections of 20 μm thickness. Mount the sections on glass slides and stain with HPS for histological analysis. The teratoma formed from pluripotent RbESCs will contain derivatives of the three embryonic germ layers.

3.9 Karyotyping

1. Day 1: Plate a suspension of 1.5×10^7 rbESCs in 30 mL medium in a 175 cm^2 flask without feeder cells.
2. Day 2: Treat rbESCs with 0.4-μg/mL colcemid solution for 4 h at 37 °C to have the cells arrested in metaphase (*see* **Note 2**). Collect the loosely attached mitotic cells by vigorously shaking the flask. Centrifuge the cell suspension at 300 g for 5 min, resuspend the pellet in 2–3 mL of warm shock solution, and incubate the cells at 37 °C for 3 min. Add 2–3

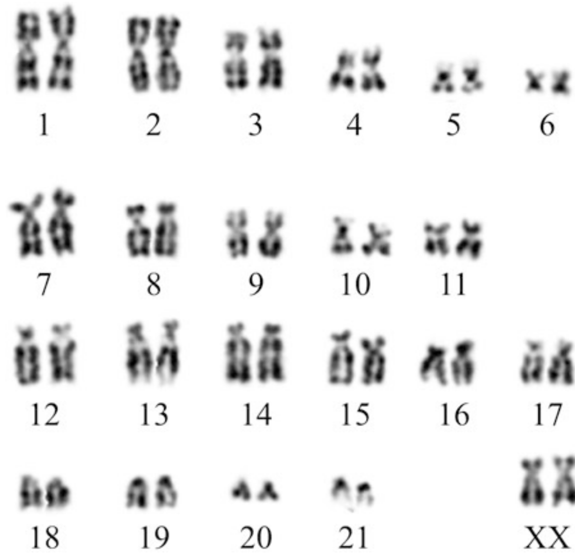

Fig. 6 G-banding karyotype of a rbESC line

drops of cold fixation solution and centrifuge the cells again at 300 g for 5 min. Resuspend the cell pellet in 200 μL of shock solution and slowly add 1–2 mL of cold fixation solution. Store the fixed cells at 4 °C.

3. Day 3: Allow 1–2 drops of a cold suspension of fixed cells to fall from a height of 20–30 cm onto an ice-cold and wet glass slide. Add 4–5 drops of fixation solution to the surface of the slide. Dry the slide on a gas flame. Incubate the slides overnight in an oven at 60 °C and store the slides at room temperature.
4. Stain the chromosome spreads by incubating the slides in Giemsa staining solution for 8–10 min. Rinse the slides twice with water and allow the slides to dry. Observe the slides under a microscope fitted with a 63× objective. Count the chromosomes of at least 50 metaphase spreads.
5. For G-banding and karyotyping, digest the chromosome spread by placing the slides in a bath of 0.045 % trypsin for 75 s at 37 °C. Rinse the slides with PBS and stain the chromosomes as described before. Observe the chromosome spreads, perform pair matching, and define the chromosomal rearrangements. An example of an euploid 42 XX karyotype is provided in Fig. 6.

3.10 Freezing and Thawing MEFs and rbESCs

1. For freezing the cells, dissociate them using 1× trypsin, count the cells, and centrifuge the suspension at 300 g for 5 min. Resuspend the cells in an appropriate volume of culture medium to obtain a cell density of 8×10^6 cells/mL. Add an equal volume of cold freezing medium dropwise into the

suspension. Distribute 0.5 mL each of the resulting suspension into separate Nunc cryotubes (2×10^6 cells/0.5 mL/tube). Place the tubes in a Nalgene cell freezing container filled with isopropanol. Place the container at −80 °C for 24 h and store the cryotubes in liquid nitrogen.

2. For thawing the cells, retrieve a vial of cryopreserved cells from liquid nitrogen and place it in a water bath for 2 min at 37 °C. Transfer the cell suspension into a 15-mL Falcon tube. Add 10 mL of warm culture medium dropwise into the tube. Centrifuge the cell suspension for 5 min at 300 g, discard the supernatant, and resuspend the cell pellet in 3–5 mL of fresh medium. Plate the cells (2×10^6 cells) onto appropriate culture plates with or without feeder cells (one 35-mm dish with feeder cells for rbESCs and 2–3 100-mm culture dishes without feeder cells for MEFs). Depending on the cell type, incubate the cells in two- or tri-gas incubator at 37 °C or 38 °C.
3. In contrast to MEFs, a high proportion of rbESCs undergo cell death after thawing. To remove the dead cells and debris, 1 day after thawing, rinse the cells twice with PBS. Add fresh medium and culture the cells for 5–7 days before passaging, daily replacing the spent medium with fresh medium. Cells should recover after two or three passages (*see* **Note 3**).

4 Notes

1. Primary MEFs are prepared from 12.5-day-old OF1 mouse embryos. Collect the uterine horns from the sacrificed pregnant mouse and isolate the implanted embryos by dissecting the horns. Rinse the embryos with PBS and remove the head and the viscera with the help of a scissor. Mince the embryo bodies with a scalpel and incubate in 5 mL of 5× trypsin for 10 min at 37 °C under constant stirring. Transfer the supernatant into a 50-mL Falcon tube containing 25 mL of fibroblast culture medium to stop the enzymatic reaction. Repeat the digestion step on the embryo pieces two more times with 5 mL of 5× trypsin. Centrifuge the dissociated cells for 10 min at 450 g. Resuspend the cell pellet in an appropriate volume of fibroblast medium. Plate 10 mL of the cell suspension onto each of the 100-mm culture dishes (same number of plates as that used for treating embryos). After 2–3 days, passage the confluent cultures in each plate into three new plates using 1× trypsin. Trypsinize the cells after 2–3 days of culture, count, and freeze at a density of 2×10^6 cells/0.5 mL freezing medium (at 10 % final concentration of DMSO). Alternatively, commercial MEFs isolated from the strain CF1 could be used (SCRC-1040, ATCC).

2. After 3 h of incubation with colcemid, every 30 min, determine the number of metaphasic rbESCs that appear round and refractive. To avoid the compaction of chromosome that will render the G-banding impossible, samples should be incubated for less than 5 h. The choice of incubation time should be a compromise between the number of blocked cells and the quality of metaphasic chromosomes.
3. To avoid spontaneous differentiation of rbESCs, it is essential to strictly follow the culture conditions described, namely, the feeder cell concentrations, the daily change of medium, and the regular passaging (once every 3–4 days).

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Gene Transfer into Pluripotent Stem Cells via Lentiviral Transduction

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Abstract

Recombinant lentiviral vectors are powerful tools to stably manipulate human pluripotent stem cells. They can be used to deliver ectopic genes, shRNAs, miRNAs, or any possible genetic DNA sequence into diving and nondividing cells. Here we describe a general protocol for the production of self-inactivating lentiviral vector particles and their purification to high titers by either ultracentrifugation or ultrafiltration. Next we provide a basic procedure to transduce human pluripotent stem cells and propagate clonal cell lines.

Keywords: Primed pluripotent stem cells, Human pluripotent stem cells, Lentivirus production, Transduction, Selection of cell clones

1 Introduction

Over the past decade, recombinant lentiviral vectors have been established as powerful tools for transgene delivery in the research fields of neuroscience, hematology, developmental biology, stem cell biology, and gene therapy (1). Lentiviruses pseudotyped with the protein of vesicular stomatitis virus VSV-G are perhaps the most versatile retrovirus since they are able to stably transduce almost any mammalian cell type, including dividing and nondividing cells, stem cells, and primary cell cultures (2). In stem cell research lentiviral vector systems are valuable tools to manipulate differentiation processes by transgene expression, to identify a favored phenotype differentiated cell populations by the use of specific promoter/reporter gene constructs (linage tracing) (3, 4) or to localize transplanted differentiated stem cells in a graft after transplantation in vivo (5).

The lentiviral vector system described herein is based on HIV-1. For the production of vector stocks, it is mandatory to avoid the emergence of replication-competent recombinants. Therefore several modifications in the wild-type HIV-1 genome were introduced into the vector system. For biosafety reasons six (*env*, *vif*, *vpr*, *vpu*, *nef*, and *tat*) of the nine HIV-1 genes were deleted from the vector system without impairing its gene-transfer ability. In the third

generation of lentiviral vector systems, attuned towards clinical applications, only *gag*, *pol*, and *rev* genes are still present, using a chimeric 5′ LTR (long terminal repeat) to enable transcription in the absence of Tat (6). In the so-called self-inactivating (SIN) vector design the U3 of 3′ LTR, which is essential for the replication of a wild-type retrovirus, is additionally deleted; thus SIN vectors cannot reconstitute their promoter and are safer than their counterparts with full-length LTRs (7, 8). On the other hand additional viral elements like the Woodchuck Hepatitis virus posttranscriptional regulatory element (WPRE) and central polypurine tract (cPPT) have been added to improve the transcriptional efficiency, which is especially important for the transduction of embryonic stem cells. The WPRE increases the overall levels of transcripts both in producer and target cells, hence increasing titers and transgene expression (9). The cPPT sequence facilitates nuclear translocation of the pre-integration complex and enhances transduction efficiency (10).

Although the latest versions of lentiviral vector system comprise four plasmids and represent the system of choice for clinical gene therapy approaches for most research applications it is adequate and more efficient to use a three-plasmid vector system with an all-purpose packaging plasmid, such as the psPAX2.

For transgene expression the choice of the promoter, which initiates the transcription, is of particular importance since it is known that widely used viral promoters, like the cytomegalovirus (CMV) promoter, can be efficiently silenced in embryonic stem cells (11). Therefore tissue-specific promoters or tetracycline-inducible elements for regulated gene expression are in most cases the promoter of choice (12, 13).

2 Materials

2.1 Cell Culture

2.1.1 Cultivation of HEK-293T Cells

1. 293T human embryonic kidney cell line (*see* **Note 1**).
2. Dulbecco's modified Eagle medium with 4.5 g/l glucose.
3. Glutamine.
4. Pyruvate.
5. Penicillin/streptomycin.
6. Fetal bovine calf serum (FBS).
7. PBS w/o Ca^{2+}/Mg^{2+} (PBS).
8. 0.25 % Trypsin/EDTA in PBS.
9. Cell culture-grade plastic plates, dishes, or flasks.

2.1.2 Feeder-Free Cultivation of Human Pluripotent Stem Cells

1. Matrigel hESC-qualified matrix (*see* **Note 2**) (Corning, Amsterdam, The Netherlands, cat # 354277).

2. mTeSR™1 culture medium (*see* **Note 3**) (STEMCELL Technologies, Vancouver, Canada, cat # 05850).
3. Knockout DMEM/F-12 (Life Technologies, Darmstadt, Germany).
4. Penicillin/streptomycin.
5. Dispase (1 U/ml) (STEMCELL Technologies, Vancouver, Canada, cat # 07923).
6. Cell culture-grade plastic plates, dishes, or flasks (e.g., 6-well plates, Corning/Falcon, cat # 353046).
7. Parafilm M.
8. PBS w/o Ca^{2+}/Mg^{2+} (PBS).
9. ROCK inhibitor, Y-27632 (Selleck Chemicals, Munich, Germany, cat # S1049).

2.2 Production, Purification, and Titer Determination of Lentiviral Vector Particles

2.2.1 Production of Lentiviral Vector Particles

1. Plasmids (available from www.Addgene.org) (*see* **Note 4**): Packaging plasmids: e.g., psPAX2 (encoding Gag, Pol, Tat, and Rev proteins) or pMDLgag/polRRE (encoding the HIV-1 Gag and Pol proteins) and pRSVrev (encoding the HIV-1 Rev protein). Envelope plasmid: pMD2G (encoding the VSV G envelope protein). Transfer plasmids: e.g., pRRLSIN.cPPT.PGK-GFP. WPRE containing your GOI (gene of interest), pLenti X1 Puro DEST, or pLenti PGK Hygro DEST (third-generation transfer vectors) (Fig. 1).
2. 293T human embryonic kidney cell line (*see* **Note 1**).
3. Routine 293 cell growth medium consisting of Dulbecco's modified Eagle medium (4.5 g/l glucose), glutamine, pyruvate, and penicillin/streptomycin with 10 % FBS.
4. 293 cell growth medium (Dulbecco's modified Eagle medium (4.5 g/l glucose), glutamine, pyruvate, and penicillin/streptomycin) but supplemented with 2 % FBS (*see* **Note 5**).
5. 2 M $CaCl_2$ solution (*see* **Note 6**).
6. 2× HEPES-buffered saline (HeBS), pH 7.00 (*see* **Note 7**).
7. 0.22 μm nitrocellulose filter.
8. PBS, pH 7.4.
9. Trypsin/EDTA, same as above.
10. 10 cm tissue culture dishes.
11. Vortex.

2.2.2 Purification of Lentiviral Vector Particles by Ultracentrifugation

1. 15 and 50 ml conical centrifuge tubes, sterile.
2. Centrifuge with swinging bucket rotor for 50 ml tubes.
3. 50 ml syringes and 0.45 μm PVDF filters.
4. 20 % sucrose solution.

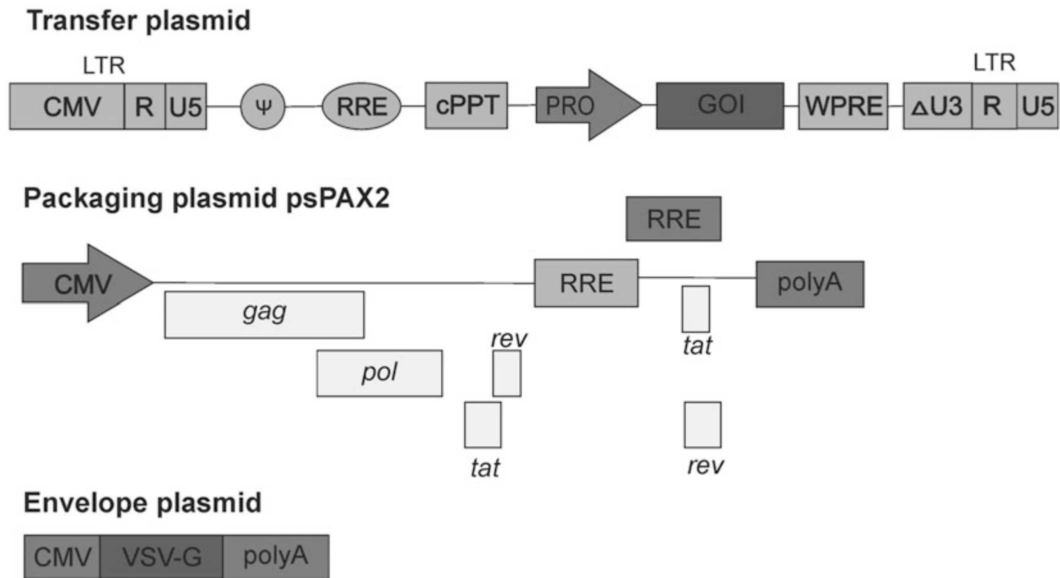

Fig. 1 Scheme of a second-generation HIV-1 vector system. The packaging plasmid psPAX expresses only the structural and regulatory HIV-1 proteins. All accessory lentiviral genes are removed. The transfer plasmid contains a self-inactivating (SIN) deletion in the U3 sequence of the 3′LTR. The Tat-dependent element in the 5′LTR is exchanged with the CMV promoter. The envelope plasmid encodes for the vesicular stomatitis virus glycoprotein, which allows the generation of pseudotyped lentivirus with broad tropism. *Psi* HIV-1 packaging signal, *RRE* rev-responsive element, *cPPT* central polypurine tract, *WPRE* woodchuck hepatitis virus posttranscriptional regulatory element, *LTR* long terminal repeat, *DU3* self-inactivating deletion of the U3 part, *Prom* promoter, *GOI* gene of interest, *Poly A* polyadenylation signal

5. 30 ml ultracentrifugation tubes (e.g., Beckman-Coulter cat # 358126).
6. Ultracentrifuge with swinging bucket rotor (50,000 × *g*, e.g., Beckman-Coulter SW28 rotor).
7. Sterile 1.5 ml microcentrifuge tubes.

2.2.3 Purification of Lentiviral Vector Particles by Ultrafiltration (Alternative Protocol)

1. 15 and 50 ml conical centrifuge tubes, sterile.
2. Centrifuge with swinging bucket rotor for 50 ml tubes.
3. 50 ml syringes and 0.45 μm PVDF filters.
4. Amicon Ultra ultrafiltration cartridges (Merck-Millipore, cat # UFC910096).
5. Sterile 1.5 ml microcentrifuge tubes.

2.2.4 Virus Titration by qPCR

1. Target cell line: e.g., HT-1080 cells (www.attc.org, cat # CCL-121) or human pluripotent stem cells.
2. DMEM medium, same as above.
3. Trypsin/EDTA, same as above.

4. 6-well tissue culture plates.
5. PBS, same as above.
6. Real-time PCR machine (Applied Biosystems, ABI ViiA7 PCR System, or equivalent).
7. Genomic DNA extraction kit (Macherey-Nagel, NucleoSpin Tissue, Düren, Germany).
8. 2× Reaction buffer (ThermoScientific, ABsolute qPCR Low ROX Mix, cat # AB-1319/A).
9. 96-well optical reaction plate (Applied Biosystems, cat # 4306737).
10. Optical caps (Applied Biosystems, cat # N801-0935).
11. Probe and primers for quantification of a common HIV-1-based lentiviral vector DNA sequence (primer: FPLV2, RPLV2; probe: LV2 (FAM/TAMRA labeled)).
    Probe and primer for quantification of the human actin beta genomic sequence (primer fw hActb, primer rv hActb, probe hActb (Yakima Yellow labeled)) (*see* **Note 8**).

2.3 Lentiviral Transduction of Human Pluripotent Stem Cells

1. Matrigel hESC-qualified matrix (*see* **Note 2**).
2. Purified lentiviral particles (*see* Sections 2.2 and 3.2).
3. mTeSR™1 culture medium (*see* **Note 3**).
4. Knockout DMEM/F-12.
5. Cell culture-grade plastic plates, dishes, or flasks.
6. Polybrene (stock solution 10 mg/ml in water).
7. ROCK inhibitor, Y-27632.
8. Antibiotics.
9. Dispase, trypsin/EDTA (same as above), or a nonenzymatic passaging solution.

3 Methods

3.1 Cell Cultivation

3.1.1 Cultivation of HEK-293T Cells

The cultivation of the human embryonic kidney cell line HEK-293T (*see* **Note 1**) is performed following standard cell culture procedures. In the following the cultivation is only briefly described.

1. The culture should never overgrow.
2. Remove the old medium from a confluent culture and wash twice with PBS to remove dead cells and cell debris.
3. Add trypsin/EDTA (TE) to the culture and incubate the cells for 3–5 min in the incubator (37 °C, 5 % CO_2).

4. To stop the trypsin reaction, add routine 293 cultivation medium (twice the volume of TE) to the well (Dulbecco's modified Eagle medium (4.5 g/l glucose), glutamine, pyruvate, and penicillin/streptomycin with 10 % FBS).
5. Transfer the cells into a conical tube and count the cells.
6. Seed 5×10^5 cells per 75 cm^2 flask and cultivate them until they reach ideal confluency for passaging. Normally, these cultures will be ready for passaging after 6–7 days. Perform a medium change at day 3 and 5 to ensure good growth conditions.

3.1.2 Feeder-Free Cultivation of Human Pluripotent Stem Cells

The coating of the cell culture plastic with Matrigel is performed according to the supplier's instructions. The following points briefly describe the coating procedure:

Preparation of Matrigel-Coated 6-Well Plates

1. To coat the cell culture plastic thaw one aliquot of the hESC-qualified Matrigel (*see* **Note 2**) slowly on ice (~1–2 h), and dilute it in a conical 50 ml tube that contains the required volume of cold knockout DMEM/F-12. Ideally, keep the conical tube during whole preparation time on ice.
2. Add the required volume of diluted Matrigel (from the conical tube) to the cell culture plastic and ensure that the whole surface is covered with a liquid film. The prepared plates should be sealed with parafilm to avoid evaporation and can be stored for up to 7 days at 4 °C in the fridge (*see* **Note 10**).
3. To ensure polymerization of the Matrigel, cell culture plates must be kept for 30–60 min at room temperature (*see* **Note 11**).
4. The remaining supernatant has to be removed prior to passaging of cells. Ensure that the well is not drying out by adding a sufficient amount of the required cultivation medium to cover the whole surface of the cavity. Now the cell culture plastic can be used for cultivation of human pluripotent stem cell (PSC) either in cell clusters or single cells.

Enzymatic Passaging of Human Pluripotent Stem Cells Using Dispase

1. When the PSC colony size is appropriate for passaging (ideally 70–90 % confluency) aspirate the medium from the cavity and wash it once with knockout DMEM/F-12 to remove dead cells and cell debris (2 ml per well of a 6-well plate).
2. Add 1 ml dispase (1 U/ml) to each well of a 6-well plate and incubate the plate for 7–10 min in the incubator (37 °C, 5 % CO_2) (*see* **Note 12**).
3. Wash twice with 1.5–2 ml knockout DMEM/F-12. After removing the knockout DMEM/F-12 add 1 ml mTeSR™1 medium (*see* **Note 13**) per well.
4. Detach the colonies with a cell scraper or a 1 ml pipette tip by washing/scraping and collect the cell clusters with a wide bore cell safer tip. During this step take care not to disrupt or

dissociate the colonies into very small fragments or even single cells because it will decrease the passaging efficiency (*see* **Note 14**). Optionally, the wells can be washed with additional medium to collect the remaining cell clusters.

5. Transfer the fragments into a conical 15 ml tube by using a wide bore cell safer tip, adjust to the required amount of mTeSR™1 culture medium, and seed the fragments onto the freshly prepared Matrigel-coated 6-well plate. Routinely, the cells can be passaged every 5–7 days with a ratio of 1:3 to 1:10 depending on the respective human PSC line. To increase the cell reattachment rate you may optionally adjust the mTeSR™1 culture medium to 10 μM ROCK inhibitor (Y-27632) for the first 24 h (*see* **Note 15**).
6. Perform a daily medium change until next passaging.

3.2 Production, Purification, and Titer Determination of Lentiviral Vector Particles

3.2.1 Production of Lentiviral Vector Particles

1. Cultivate 293T cells in DMEM medium on 10 cm tissue culture dishes in a 37 °C humidified incubator with a 5 % CO_2 atmosphere.
2. The day before the transfection, seed the required number of dishes at 2.5 million cells per dish (10 cm). Incubate overnight in a 37 °C humidified incubator with a 5 % CO_2 atmosphere. On the following day, co-transfect the cells by calcium phosphate precipitation according to the following recipes (Fig. 2).
3. For one 10 cm dish mix 5 μg of envelope plasmid, 10 μg of packaging plasmid, and 12 μg of vector plasmid containing your gene of interest (GOI) and adjust to 438 μl with the water in a 15 ml tube (*see* **Note 9**) (Fig. 1).
4. Add 62 μl of 2 M $CaCl_2$.
5. Mix well and add this mix dropwise and slowly (one drop every second) to 500 μl of 2× HeBS, while vortexing with top speed.

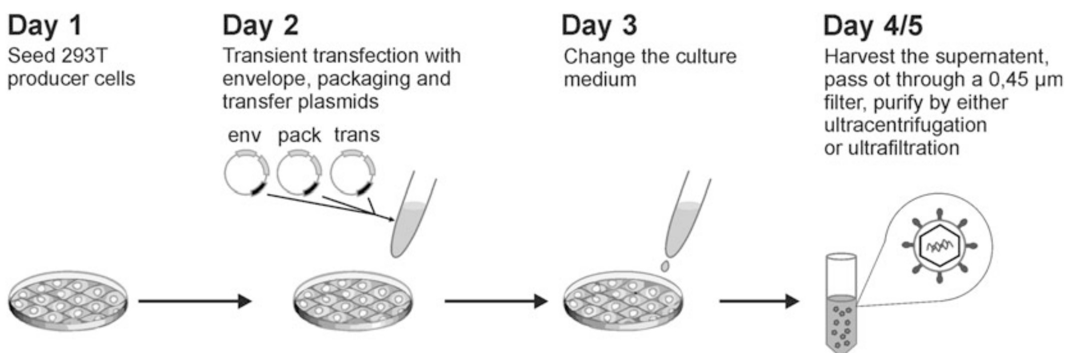

Fig. 2 Schematic presentation of the production of lentivirus vector particles. Details of the production procedure are outlined in Section 3.2

6. Let stand still for 20 min (not longer than 30 min) on the bench.
7. Just prior to transfection of 293T cells, remove the medium and gently add fresh 293 DMEM medium.
8. Add the precipitate slowly dropwise on the cell monolayer. Shake (not stir) the plate gently (*see* **Note 16**).
9. Incubate the dishes overnight in a 37 °C humidified incubator with a 5 % CO_2 atmosphere.
10. On the next day discard the medium after gentle, but firm, stirring (to eliminate as much precipitate as possible), and replace with 10 ml of fresh and pre-warmed medium supplemented with 10 % or 2 % FBS (*see* **Note 17**) (Fig. 2).
11. Discard the medium after gentle, but firm, stirring (to eliminate as much precipitate as possible) and replace with 10 ml of new medium containing 10 mM sodium butyrate.
12. On day 4, harvest the supernatant and replace with 10 ml DMEM medium supplemented with 10 % or 2 % FBS (Fig. 2).
13. Collect the supernatant in a 50 ml tube and pellet detached 293T cell at 500 × *g* for 5 min at +4 °C.
14. Transfer the supernatant to a new 50 ml tube and filter the fluid through 0.45 μm PVDF filter to remove remaining cell debris. Store the virus preparation at +4 °C (*see* **Note 18**) (Fig. 2).
15. Additional lentiviral particles can be harvested after 24 h later by repeating the procedure.
16. For determination of the biological titer seed 1×10^5 target cells on 24-well plates.

3.2.2 Lentivirus Concentration by Ultracentrifugation

1. For the concentration use 30 ml conical tubes (e.g., Beckman-Coulter cat # 358126,) with an SW 28 rotor in an ultracentrifuge. Sterilize the centrifuge tubes through incubation with 70 % ethanol for 5 min. Remove the ethanol, and place the tubes into a laminar flow hood with UV light on for 30 min for drying.
2. Fill 4 ml of 20 % sucrose on the bottom of the tube. Pour the purified virus solution from Section 3.2.1 very slowly on the surface of the sucrose cushion until the tube is completely filled. Spin at 25,000 rpm (82,700 × *g*) for 2 h at +4 °C.
3. Carefully remove the tubes from the rotor, pour off the supernatant, and leave tubes on a paper towel in an inverted position for 10 min to allow the residual liquid to drip away from the pellet.
4. Place the conical tube in a 50 ml Falcon tube and quickly add 30–100 ml of PBS on the pellet (not always visible) (*see* **Note 19**).
5. Close the Falcon tube. Incubate the tubes at +4 °C for 2 h. Vortex very gently every 20 min.

6. Spin the tubes at 500 × *g* for 1 min to collect the virus-containing liquid.
7. The concentrated virus solution can be stored at −80 °C for at least 1 year.

3.2.3 Lentivirus Concentration by Ultrafiltration

1. If no ultracentrifuge is available lentiviral vector particles can be concentrated by ultrafiltration (e.g., Merck-Millipore, Amicon Ultra Ultracel 100 K, cat # UFC910096).
2. Add 15 ml H_2O to the filter cartridge according to the manufacturer's manual and spin at 3,000 × *g* for 5 min.
3. To sterilize the filter cartridge add 15 ml 70 % ethanol and incubate for 2 min (*see* **Note 20**).
4. Discard the ethanol from the reservoir, dry the filter by centrifugation at 3,000 × *g* for 5 min, and remove the flow through.
5. Pipette 15 ml of the purified virus solution from Section 3.2.1 on the filter cartridge and centrifuge at 3,000 × *g* at +4 °C for approx. 20 min. The volume of the concentrated virus solution should be between 160 and 200 μl.
6. The concentrated virus solution can be stored at −80 °C for at least 1 year.

3.2.4 Titer Determination of Lentiviral Vector Particles

1. For the determination of the biological titer, seed 10^5 HT-1080 cells or 10^6 human PSCs per well on a 6-well plate.
2. After 24-h cultivation trypsinize 1 well and count the number of cells.
3. Aspirate the medium of the wells and add 2 ml fresh medium. Transduce the cells typically with 1:100, 1:1,000, and 1:10,000 dilutions of the concentrated virus per well.
4. Cultivate the cells overnight in a 37 °C humidified incubator with a 5 % CO_2 atmosphere.
5. Aspirate the culture medium and add 5 ml DMEM medium or 3 ml mTeSR™1 (for human PSCs).
6. Incubate the cells for additional 2 days in a 37 °C humidified incubator with a 5 % CO_2 atmosphere.
7. Trypsinize the cells and extract target cell DNA from each individual well plate using a genomic DNA extraction kit following the manufacturer's recommendations. Elute the DNA in 100 μl elution buffer.
8. DNA samples from the transduced cells are measured in triplicates. Prepare qPCR master mix for the desired number of samples as follows:

Reaction buffer ABsolute qPCR	
Low ROX Mix 2×	5
Primer fw LV	0.5 × *n*
Primer rev LV	0.5 × *n*
Primer fw hActb	0.5 × *n*
Primer rev hActb	0.5 × *n*
Probe LV2 (10 μM, FAM labled)	0.2 × *n*
Probe hActb (10 μM, Yakima Yellow labled)	0.2 × *n*
H_2O	0.1 × *n*
Total	7.5 × *n*

n = number of reactions

9. Pipette 7.5 μl of the master mix per well on the 96-well optical reaction plate.
10. Add 2.5 μl template solution (genomic DNA of transduced cells) per well.
11. Seal the wells with optical caps, stripes, or foils, vortex the plate gently, spin down the liquid, and put the plate into the qPCR machine.
12. The amplification is performed with the following program: 95 °C for 15 min, 95 °C for 20 s, 60 °C for 2 min.
13. For the duplex Taqman assay the FAM and Yakima Yellow fluorescence is detected by the qPCR device.
14. Data analysis: Vector copy numbers in HT-1080 cell or human PSCs are normalized to human actin beta gene copies and presented as proviral copies per genome equivalent. Calculate titers (IP = infective particles per ml) according to the following formula:

    $IP/ml = (C \times N \times D \times 1{,}000)/V$ (C = proviral copies per genome, N = number of cells at time of transduction, D = dilution of vector preparation, V = volume of diluted vector added in each well for transduction).
15. To determine an accurate titer calculate mean values from at least two vector dilutions.

3.3 Lentiviral Transduction of Human Pluripotent Stem Cells

3.3.1 Lentiviral Transduction of Human PSC Colonies

1. Passage the human PSCs as clumps onto a Matrigel-coated 6-well plate as described in Section 3.1.2. Cultivate the cells in mTeSR™1 medium until the colonies are recovered from the last passaging procedure and reached a good size for transduction. In our hands the best results were obtained when the colonies were approximately 3–4 days prior to the next passaging (Fig. 3).

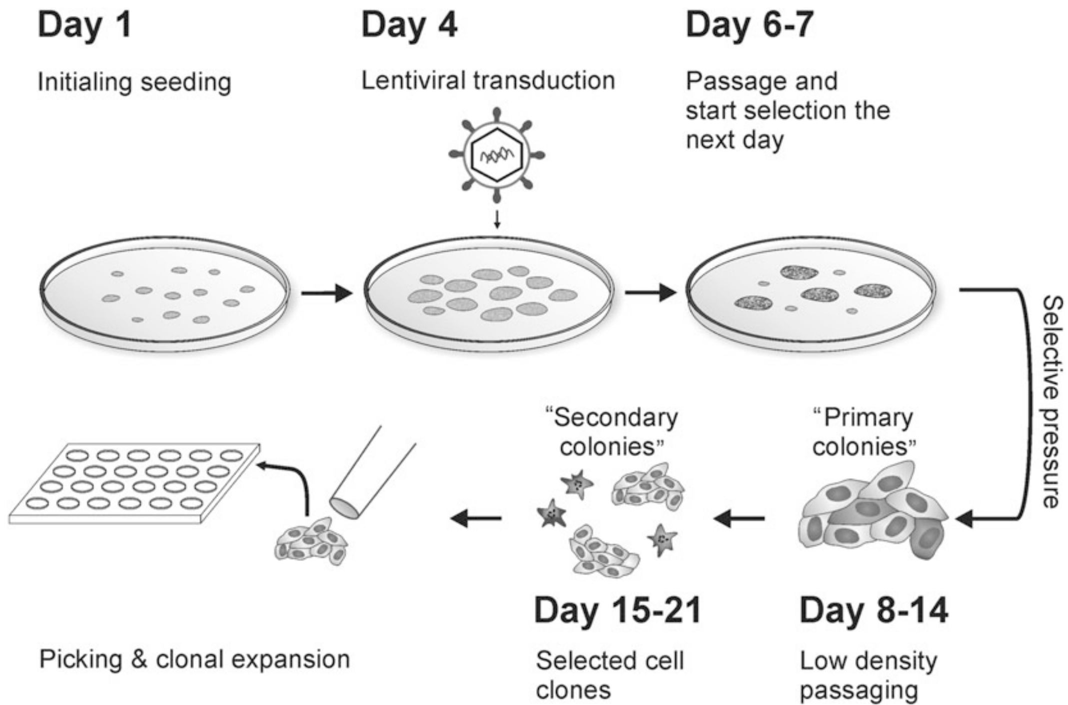

Fig. 3 Schematic presentation of the transduction and selection of pluripotent stem cells. Details of the procedure are outlined in Section 3.3

2. Thaw the concentrated lentiviral solution (produced according to Section 3.2) on ice.
3. For 1 well of a 6-well plate add the volume of the concentrated lentiviral solution with the desired amount of IP, move the plate to equally distribute the lentiviral solution, and add 1 ml mTeSR™1 medium. To increase the transduction efficiency 5–10 μg/ml polybrene (1:1,000–2,000 dilution from the stock solution) can be added.
4. Place the cells back into the incubator (37 °C, 5 % CO_2) for 4–6 h. Thereafter, add one additional milliliter of mTeSR™1 medium and cultivate the cells overnight in an incubator (*see* **Note 21**).
5. If a second, optional transduction round should be performed, repeat the points 2 to 4 24–48 h after the first transduction.
6. One day after the last transduction wash the cells twice with 2 ml knockout DMEM/F-12 per well and add fresh mTeSR™1 medium for the further cultivation. Perform daily medium changes with mTeSR™1 until the culture is ready for passaging. Begin the antibiotic selection after this passaging.

3.3.2 Lentiviral Transduction of Single Cells

Human PSCs can be passaged with different methods into single cells. For this purpose enzymatic passaging (e.g., with TE) or nonenzymatic passaging solutions (e.g., gentle cell dissociation reagent from Stem Cell Technologies) can be used.

1. Wash the cells once (or twice) with PBS.
2. Dissociate the human PSCs either enzymatically or nonenzymatically into single cells. If TE is used incubation for 7–10 min at 37 °C is recommended. Stop the trypsin reaction by adding a medium supplemented with 10 % FBS, if your pluripotent cell line accepts this procedure. Alternatively, use knockout DMEM/F-12. If a nonenzymatic passaging solution is used please follow the manufacturer's instructions.
3. Collect the cells in a conical tube and break up potential remaining clusters by pipetting the cell solution up and down.
4. Centrifuge for 3–5 min at 400–700 × *g* and remove the supernatant completely.
5. Add the desired amount of mTeSR™1 medium, adjust to 10 μM Y-27632, and count the cells.
6. Seed 50,000–100,000 cells/cm^2 (*see* **Note 22**) on a 12-well or 6-well plate freshly coated with Matrigel in mTeSR™1 medium containing 10 μM Y-27632 to allow reattachment of dissociated cells. Culture for 24 h in the incubator (37 °C, 5 % CO_2).
7. At the next day thaw the concentrated lentiviral solution (produced as described in Section 3.2) on ice.
8. For transduction remove the old medium and add the desired volume of the concentrated lentiviral solution (containing the desired IP) into the well. Distribute the lentiviral solution equally by moving the plate and add 1 ml mTeSR™1 medium. Identically to the transduction of hPSC colonies the efficiency can be increased by addition of 5–10 μg/ml polybrene (1:1,000–2,000 dilution from the stock solution).
9. Place the cells for 4–6 h into the incubator (37 °C, 5 % CO_2); thereafter add one additional milliliter of mTeSR™1 medium and cultivate the cells overnight (*see* **Note 21**).
10. Optionally, a second transduction can be performed 24–48 h later by repeating points 7 to 9.
11. After the last transduction round the cells should be washed twice with 1–2 ml knockout DMEM/F-12 per well. Further cultivation is performed typically with mTeSR™1 medium. Therefore, perform daily medium changes until the culture is ready for passaging. Passage the cells thereafter as clusters as described above (Section 3.1.2).
12. Start with the antibiotic selection after this passaging procedure.

3.4 Selection, Propagation, and Clonal Derivation of Transduced Cells

The selection can be initiated already 1–2 days after the last transduction round but from our experience we recommend to split the cells once prior to selection. In addition, a kill curve should be performed for every human PSC line to determine the ideal concentration of the selective antibiotics. In the case of a fluorescent reporter positive cells may be purified by fluorescence-activated cell sorting (FACS). The following paragraph describes a general protocol for the selection via an antibiotic resistance gene.

3.4.1 Determination of the Ideal Antibiotic Concentration

1. Passage the human PSCs as described in Section 3.1.2. For a kill curve minimally 4–6 different concentrations should be tested for the specific human PSC line. Classical antibiotics for the selection are blasticidin, hygromycin, neomycin (G418), puromycin, and zeocin.
2. Begin with the treatment 2–3 days after passaging and perform daily media changes with mTeSR™1 containing the different concentrations of the selective antibiotics.
3. Check the cells under the microscope and monitor the selection progress.
4. Choose the concentration, at which the human PSCs show clear signs of a toxic effect of the selective antibiotic after 2–3 days. After this time point cell death should clearly occur. In our hands, such an ideal concentration will increase the number of positive selectable clusters/colonies after lentiviral transduction, especially if colonies were used for transduction.

3.4.2 Selection of Transduced Cell Clones

1. Passage the human PSCs transduced with the lentiviral vector particles as clusters with dispase as described under Section 3.1.2 and schematically shown in Fig. 3.
2. Begin the selection procedure 1 day after passaging with the defined concentration of the selective antibiotic (*see* Section 3.4.1) in mTeSR™1 medium.
3. Perform daily media changes with selective medium (mTeSR™1 containing the desired concentration of the selective antibiotic).
4. Typically, after 3–4 days first small colonies/clusters (or parts of a larger colony) are visible, which show no signs of a toxic effect from the selection. These small colonies will increase in size during the further cultivation (*see* **Note 23**).
5. Cultivate the transduced cell colonies until they reach a sufficient size to passage them. Ideally, the first clones should not be picked before day 7 of selection (*see* **Note 24**).

3.4.3 Clonal Expansion of Transduced hPSCs

Stably transduced cell colonies can be directly picked once they have reached a sufficient size. However, these primary colonies might have been derived from more than one transduced cell. To

minimize the risk of mixed clonal identity, it is recommended to passage primary colonies once more. Ideally, these primary colonies should be passaged into single cells in low density so that newly arising secondary colonies will be formed from a single-cell clone. However, this might not be feasible for all human PSC lines and should be experimentally tested for each PSC line. Thus, this chapter describes the procedure starting with primary colonies.

1. Once primary colonies (cell clones) have reached a passaging-ready size they are passaged in the presence of the selective antibiotic as described in Section 3.2.1 onto a Matrigel-coated 6-well plate. The ideal passage ratio depends on the obtained number of colonies. Use a high passage ratio so that only a few small clusters evenly distribute in a well (*see* **Note 23**) (Fig. 3).
2. Cultivate these clusters until they grow up to a size ideal for passaging in the presence of antibiotics in the culture medium. This takes usually 7–10 days. Under microscopical inspection using an inversed cell culture microscope mark colonies from the underside of the well with a permanent marker. Mark only colonies that leave sufficient space to neighboring colonies without direct cell contacts.
3. Each of these marked colonies can be transferred into a new well of a 24- or 48-well plate by manual picking. At the day of picking prepare the required number of wells of a 24- or 48-well plate and coat them with Matrigel. To ensure that the picked colonies have a sufficient amount of mTeSR™1 medium add approx. 0.5–0.75 ml per well.
4. Wash the wells once with knockout DMEM/F-12 to remove dead cells and cell debris (2 ml per well of a 6-well plate).
5. Add dispase (1 U/ml, 1 ml into each well of a 6-well plate) and incubate the plate for 7–10 min in the incubator (37 °C, 5 % CO_2) (*see* **Note 12**).
6. Wash twice with 1.5–2 ml knockout DMEM/F-12, remove the knockout DMEM/F-12 completely, and add 1.5–2 ml mTeSR™1 medium per well of a 6-well plate where colonies should be picked from.
7. Place the plate under the microscope and search for marked colonies. Center one colony in the field of vision. Remove the lid of the plate and use a sterile 100 μl tip (on a pipette) or a sterile cannula to cut the colony into smaller clumps under optical inspection. Collect the fragments by scraping with the 100 μl tip of the pipette over the bottom and aspirate the clumps.
8. Transfer the clumps of one colony into 1 well of the coated 24- or 48-well plate. Change the tip after each picked colony. Take care not to aspirate floating cell debris/fragments during the

picking process as this could lead to mixed clonal identity (Fig. 3).

9. After the picking place the 24- or 48-well plate containing the primary clones into the incubator (37 °C, 5 % CO_2).
10. Analyze the picking efficiency the next day under the microscope and cultivate the clones until they are ready for passaging. This takes usually 7–10 days.
11. Propagate the cell clones and immediately freeze stocks when the cell mass is appropriate.
12. For each clone a quantification of the number of lentiviral integrations, a characterization of pluripotency (minimal expression analysis and staining of key pluripotency genes or a teratoma assay), and ideally a karyotype analysis are recommended.

4 Notes

1. The HEK293T human embryonic kidney cell line is available from ATCC (cat # CRL-11268). There are also several commercial cell lines available which were selected for high virus titer production.

293 FT Cell Line (Cat. # R700-07)	Life Technologies
Lenti-X™ 293T Cell Line (Cat. # 632180)	Clontech
293LTV Cell Line (Cat. # LTV-100)	Cell Biolabs

2. The hESC-qualified Matrigel should be stored in aliquots at −80 °C following the manufacturer's instructions. After coating, the supernatant still contains matrix proteins that have not yet polymerized; thus, Matrigel may be reused but it has to be tested for each human PSC line separately. The used Matrigel can be stored at 4 °C for up to 7 days and could be used for coating just like fresh Matrigel.
3. After preparing mTeSR™1 medium it can be stored for up to 14 days at 4 °C. The required mTeSR™1 for each day should be warmed up as aliquot to ensure that the residual medium is always stored at 4 °C. To increase the shelf life it can be stored upon preparation in aliquots at −20 °C for up to 6 months and thawed in appropriate amounts.
4. The specified lentiviral transfer plasmids are only examples. Various plasmids for different applications are available from Addgene (www.addgene.org) or from commercial suppliers.

5. DMEM supplemented with 2 % FBS for virus concentration with Amicon Ultra ultrafiltration cartridges (Merck-Millipore).
6. 2 M $CaCl_2$: Dissolve 7.35 g of $CaCl_2 \times 2H_2O$ (Sigma-Aldrich cat # C5080) in 25 ml H_2O. Sterilize the solution by filtration with 0.22 μm nitrocellulose filter. Store in 2 ml aliquots at −20 °C.
7. 2 × HeBS (HEPES-buffered saline): Dissolve 16.36 g of NaCl (Sigma-Aldrich cat # S7653; 0.28 M final), 11.9 g of HEPES (Sigma-Aldrich cat # H7523; 0.05 M final), and 0.213 g of Na_2HPO_4, anhydrous (Sigma-Aldrich cat # S7907; 1.5 mM final) into 800 ml H_2O. Adjust pH to 7.0 with 10 N NaOH. It is very important to adjust to the correct pH. The precipitate will not form below 6.95. Above 7.05 the precipitate will be coarse and transfection efficiency will be low. Add H_2O to 1,000 ml, and perform the final pH adjustment. Filter sterilize through a 0.22 mm nitrocellulose. Store at −20 °C in 50 ml aliquots. Once thawed, the HeBS solution can be kept at +4 °C for several weeks.
8. The primer (FPLV2, ACCTGAAAGCGAAAGGGAAAC; RPLV2 CACCCATCTCTCTCCTTCTAGCC) and probe (LV2, 5′-FAM AGCTCTCTCGACGCAGGACTCGGC-BHQ1) sequences for detecting the copy number of the lentiviral genome were selected according to (14). Primer and probe sequences for the quantification of the human actin beta: FPhActb tcgtcgtcgacaacggct; RPhActb agagaaagcgcccttgcct; probe YY 5′-catgtgcaaggccggcttcgc-BHQ1. If no duplex assay capable for qPCR device for the parallel detection of green and yellow fluorescence dyes is available the TaqMan assay for the copy number quantification can be split into two separate reactions/wells using FAM-labeled probes.
9. If a four plasmid-based lentiviral vector system is used, 10 μg packaging plasmid (e.g., psPAX2), 7 μg pMDLgag/polRRE (encoding the HIV-1 Gag and Pol proteins), and 3 μg pRSVrev (encoding for Rev) are necessary.
10. The time for storing Matrigel can be prolonged to up to 10 days, which had in our hands no negative effects. Generally, it is recommended to prepare only the amount of Matrigel that is required within the next 7–10 days.
11. If a faster polymerization is required the cell culture plastics containing the cold Matrigel solution can be incubated for 15–20 min in the incubator (37 °C, 5 % CO_2) to polymerize quicker. Thereafter, the surface should be washed once with knockout DMEM/F-12 (room temperature) prior to seeding the passaged human PSC clusters.
12. The ideal incubation time depends on the cell line and the size of the colonies and has to be determined individually.

Generally, inspect the wells after around 7 min of incubation and look whether the colony rim lifted up (brighter rim of the colony under a phase-contrast microscope). Ideally the colony rim should be lifted up but not the core of the colony.

13. To minimize costs the cells can also be scraped and collected in 1 ml knockout DMEM/F12 instead of mTeSR™1 medium. In this case after collecting the clusters an additional centrifugation step (3 min at 300 × *g*) is required. The supernatant must be removed and the required amount of mTeSR™1 medium can be carefully added (take care not to break up the clusters too much). Now the human PSC cluster can be distributed into the new Matrigel-coated wells in the desired density.
14. If the fragments got too small cell survival will be quite low indicated by floating dead cells and cell debris on the next day. To avoid this, scrapping should be reduced to max 2–3 times over a given surface area. Collect the clusters with a wide bore cell safer tip to reduce steric stress. Still attached clusters can be washed away with 1 ml of mTeSR™1 or knockout DMEM/F12 medium.
15. Generally, the optimal passaging ratio should be determined individually for each human PSC line. The addition of ROCK inhibitor Y-27632 may result in a higher split ratio. However, in our experience prolonged passaging with Y-27632 resulted in a worsening morphology over time compared to passaging without Y-27632.
16. At this point, there should be no visible precipitation because it is too fine. If a precipitation is visible, then it is too coarse and your transfection will most likely be less efficient.
17. For the alternative concentration protocol by ultrafiltration, a reduction of the FBS supplementation to 2 % is recommended for an optimal filtration result. The FBS reduction might slightly reduce the virus titer.
18. The lentivirus stocks can be stored at +4 °C for 1–4 days without significant titer loss before they are used for transduction of target cells or concentration. For longer storage stocks must be kept at −80 °C.
19. Do not leave the pellet dry for more than 5 min or it may result in a significant titer decrease.
20. Longer ethanol incubation may negatively affect the constancy of the filter cartridge material.
21. Alternatively, the cells can be washed twice with 2 ml knockout DMEM/F-12 after the transduction time of 4–6 h. The cultivation afterwards is typically performed with mTeSR™1 medium. This procedure is preferable if a second transduction round will be performed within the next 24–48 h.

22. The ideal cell number per cm^2 must be empirically determined and depends on the reattachment rate of the used human PSC line.
23. It may happen that some of the primary colonies will die during further selection. This is likely caused by two independent phenomena. First, if a primary colony is mixed with transduced and non-transduced cells, later cells will start to deteriorate causing a disintegration of the whole colony. Second, PSCs are known for epigenetic silencing of transgenes especially of ectopic viral DNA sequences such as viral promoters or other regulatory elements. Silencing takes place very rapidly and may cause loss of resistance against the selection. Thus, our recommendation is to passage transduced cells once before selection to increase the likelihood that the transduced cells are able to form a clonal cell cluster and to keep up the selective pressure by adding antibiotics to the culture medium. However, it might be useful to reduce the antibiotic concentration after 1 week of selection.
24. Cell clones may be passaged under selective pressure. Residual non-transduced cells are additionally destroyed and ongoing selection reduces the risk of transgene loss/epigenetic silencing. However, the reattachment rate after the first passaging is lower compared to passaging without selection pressure. Generally, the minimal selection time should be around 14–21 days. In our hands no negative effects were detectable upon permanent culture in the presence of the selective antibiotic.

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Imaging Pluripotency: Time-Lapse Analysis of Mouse Embryonic Stem Cells

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Abstract

The current view of the pluripotent state is that of a transient, dynamic state, maintained by the balance between opposing cues. Understanding how this dynamic state is established in pluripotent cells and how it relates to gene expression is essential to obtain a more detailed description of the pluripotent state.

In this chapter, we describe how to study the dynamic expression of a core pluripotency gene regulator—Nanog—by exploiting single-cell time-lapse imaging of a reporter mESC line grown in different cell culture media. We further describe an automated image analysis method and discuss how to extract information from the generated quantitative time-course data.

Keywords: Stem cells, Nanog, Pluripotency, Heterogeneity, Dynamics, Time-lapse imaging

1 Introduction

Mouse Embryonic stem cells (mESCs) are pluripotent cells derived from the inner cell mass of the blastocyst. They are capable of both self-renewing and multilineage differentiation, enabling the regulation of cell number and type during early embryonic development (1, 2). Due to their properties, ESCs hold great potential for medical application (3, 4). However, to fully exploit this potential a conceptual understanding of the pluripotent state is required.

Pluripotency is controlled by a gene regulatory network (GRN) of transcription factors, with the triplet Oct4, Nanog, and Sox2 forming its core (5). They function together in activating other pluripotency genes, while repressing differentiation-promoting genes (6, 7). Oct4 and Sox2 can also drive the production of FGF4, a differentiation factor, functioning as lineage specifiers. In contrast, Nanog overexpression is able to suppress differentiation and maintain pluripotency even in the presence of FGF4

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signaling. Thus, the current picture of the pluripotent state is that of a transient, dynamic state, maintained by the balance between opposing cues (8).

Contrasting with homogenous levels of OCT4 and SOX2, NANOG expression in mESCs is heterogeneous (9, 10), with a bimodal distribution between high- and low-expressing subpopulations. Possibly, these subpopulations have different responsiveness to differentiation stimuli (11, 12). This would allow keeping a pool of progenitors cells even in differentiating conditions.

NANOG levels fluctuate in individual cells; however, the source of this "noise" and its role remains unclear (13). One hypothesis is that fluctuations prime individual cells for differentiation, without marking definitive commitment. Different culture conditions (promoting self-renewal or differentiation) shift this equilibrium, without suppressing fluctuations. Understanding how this dynamic behavior is established in mESCs and how it relates to gene expression will lead to a more detailed description of the pluripotent state.

To investigate the dynamics of Nanog heterogeneity in mESCs, it is crucial to perform real-time monitoring of its expression in individual cells. This requires the use of a faithful reporter with good correlation between the temporal expression of reporter and endogenous proteins. Previously, we have established a NANOG reporter cell line (Nd) (10) containing a transgenic Bacterial Artificial Chromosome carrying a cDNA coding for a bright and fast degrading yellow fluorescent protein (VNP), inserted under the control of the Nanog regulatory regions. Our data show that the VNP reporter can be used as a faithful proxy for NANOG dynamic expression, and we have now improved this cell line to allow automated tracking, by introducing a constitutive mCherry reporter that is homogeneously expressed by all cells (NdC, from Nd plus mCherry).

In this chapter, we describe protocols to perform time-lapse fluorescence microscopy of single NdC cells expressing the Nanog fluorescent reporter (Section 3.2) under different self-renewing culture conditions (Section 3.1). We also describe a MATLAB-based software developed for automated image analysis (cell segmentation and tracking) (Section 3.3). These protocols provide a rapid and reliable way to follow in real-time fluorescent fluctuations of the reporter and extract information on the dynamic behavior of NANOG (Section 3.4), such as frequency and duration of expression, and amplitude of fluctuations.

2 Materials

Prepare all solutions under sterile conditions, in a laminar-flow cell culture hood (class II). mESCs should be preserved in cryogenic storage, with 10 % of DMSO as protective agent.

2.1 mESC Lines

The following mESC lines have been used:

1. E14tg2a cells, not genetically modified mESC line derived from 129/Ola mice blastocysts (a kind gift from Austin Smith's lab, University of Cambridge, UK).
2. NdC cells, a novel NANOG reporter mESC line derived from Nd mESCs (10) by stable integration of a mCherry-NLS reporter, which is expressed in all cells' nuclei.

2.2 Media and Reagents

All reagents stored at −20 °C should be removed from the freezer and leave to thaw overnight at 4 °C before use.

2.2.1 mESC Culture Media

1. Serum/LIF conditions:
   (a) Mix the following components in a 250-ml bottle:
      - 200 ml of sterile of 1× Glasgow Modified Eagle's medium (GMEM, GIBCO).
      - 2 ml of 200 mM glutamine (100×, GIBCO, stored in aliquots at −20 °C).
      - 2 ml of 100 mM Na pyruvate (100×, GIBCO, stored in aliquots at −20 °C).
      - 2 ml of 100× nonessential amino acids (GIBCO, stored at 4 °C).
      - 2 ml of 100× penicillin–streptomycin solution (GIBCO, stored in aliquots at −20 °C).
      - 200 μl of 0.1 M 2-mercaptoethanol (Sigma). The stock solution should be prepared in sterile ultrapure water, stored at 4 °C and used within 4 weeks).
      - 20 ml of fetal bovine serum (GIBCO ES-qualified FBS, inactivated 30 min at 55 °C and stored in aliquots at −20 °C).

   (b) Filter through a 0.2 μm filter unit into a new sterile flask, store at 4 °C and use within 1 month.
   (c) Supplement with 2 ng/ml Leukemia inhibitory factor (LIF) prior to use.
2. 2i/LIF conditions:
   (a) Supplement iStem medium (Stem Cells Inc.) (8) with respective supplement, store at 4 °C and use within 2 weeks. iStem media and respective supplement should be aliquoted, stored at −20 °C and always protected from light.
   (b) Supplement with 2 ng/ml LIF prior to use.

2.2.2 Poly-L-ornithine and Laminin Coating

1. Incubate plates with 1 μg/ml poly-L-ornithine/H_2O (Sigma) for 20 min at room temperature.
2. Wash twice with PBS.

3. Incubate with 10 μg/ml laminin (Sigma) in PBS for at least 3 h at 37 °C.
4. Aspirate laminin just before plating the cells.

2.2.3 Other Reagents

1. 0.1× Trypsin solution:
   (a) Prepare a 1× trypsin solution by mixing: 5 ml of 2.5 % trypsin (Gibco), 0.5 ml of heat-inactivated chicken serum, 0.1 ml of 0.5 M EDTA, and PBS to 50 ml.
   (b) Dilute the 1× trypsin solution with PBS.
2. 0.1 % Gelatine: dilute from 2 % in H_2O, tissue culture grade Gelatine solution (Sigma).
3. 2× Freezing medium: mix 800 μl of 1× Serum/LIF medium with 200 μl of DMSO Hybri-max (Sigma).

2.3 Equipment

2.3.1 Materials for Tissue Culture

1. Lab-Tek™ II Chamber Slide™ System (Nunc, cat.no. 155411).
2. Glass bottom MatTek culture plates (MatTek, cat.no. P35G-1.5-14-C).
3. 6-well multiwell tissue culture dish (Nunc, cat.no. 140675).
4. 60-mm tissue culture dishes (Nunc, cat.no. 150288).

2.3.2 Microscope

The microscope station for time-lapse imaging may vary. We used a 3i Marianas spinning disk confocal microscope (https://www.intelligent-imaging.com/marianas.php). Here is a list of the necessary components:

1. Inverted microscope.
2. High numerical aperture objectives (we used 63× oil immersion, a 40× is also recommended).
3. Laser sources. For multiple color acquisition at least two different wavelengths are necessary. For example, we used 488 nm and 561 nm, or 488 nm and 640 nm to ensure proper spectral separation.
4. Emission filter set adequate to the selected imaging wavelengths.
5. Spinning disk unit.
6. High resolution, high sensitivity CCD or EMCCD camera.
7. Incubator for temperature control and CO_2 supply.
8. CO_2 supply.
9. Motorized xy stage to acquire multiple positions.
10. Piezo for z-stacks acquisition.
11. Controlling computer and data acquisition software.
12. Computer for data analysis.
13. External storage for data files.

3 Methods

3.1 mESC Culture

All cell manipulations should be performed under sterile conditions, in a laminar-flow cell culture hood (class II). mESCs are grown at 37 °C in a 5 % (v/v) CO_2 incubator.

We routinely thaw and expand mESCs in Serum/LIF media, and only change to 2i/LIF culture media for specific experiments, but routine expansion can also be performed in 2i/LIF media.

3.1.1 Thawing and Routine Expansion of mESCs

1. Thaw mESCs in Serum/LIF medium:
   - (a) Coat a 60-mm Nunc dish with 0.1 % Gelatine (for minimum of 10 min).
   - (b) Heat 10 ml of Serum/LIF medium in a 37 °C waterbath.
   - (c) Remove cells from cryogenic conditions and place in the 37 °C waterbath for approximately 1 min (medium color changes from yellowish to pink).
   - (d) Resuspend cells in 4 ml of heated Serum/LIF medium and spin cells down (2 min, 165 × *g*).
   - (e) Remove supernatant, resuspend cells in 5 ml of Serum/LIF medium and transfer to the previously gelatine-coated dish.
2. Change medium within 6–12 h.
3. Passage cells on the next day (*see* **Note 1**):
   - (a) Wash cells twice with PBS.
   - (b) Add 0.1× Trypsin (just enough trypsin solution to cover the cells) and place in the incubator for 2–3 min.
   - (c) Knock the dish several times to dissociate cells and check under inverted microscope to ensure the cells have dissociated.
   - (d) Add serum containing medium (Serum/LIF) to stop trypsinization, resuspend the cells by pipetting up and down and spin cells down (2 min, 165 × *g*).
   - (e) Resuspend cells in Serum/LIF medium, count the viable cell number using trypan blue dye exclusion method and, when appropriate, take a sample for flow cytometry analysis (Section 3.1.3).
   - (f) Inoculate cells at the desired cell density (for routine mESC expansion plate 3×10^4 cells/cm^2) in Serum/LIF medium, on freshly coated gelatine dishes.
4. Passage cells every other day (when cells reach 70–80 % confluence, *see* **Note 1**) at a constant plating density, in the desired cell culture media.

3.1.2 Plating mESCs for Time-Lapse Imaging

1. Two days before imaging, plate mESCs in Serum/LIF or 2i/LIF media as described in Section 3.1.1 (*see* **Note 2**). Plating of one well of a 6-well culture dish usually provides sufficient cells for the rest of the protocol.
2. One day before imaging, coat the required wells of a Lab-Tek Nunc chamber slide with poly-L-ornithine and laminin.
3. On the imaging day, dissociate mESCs (as described in Section 3.1.1) and count the viable cell number using trypan blue dye exclusion method.
4. Take a cell sample for flow cytometry analysis (Section 3.1.3) (*see* **Note 3**).
5. Inoculate cells at the desired cell density (2×10^4 cells/cm^2 or 3×10^4 cells/cm^2, respectively for Serum/LIF and 2i/LIF media, *see* **Note 4**) on poly-L-ornithing and laminin (*see* **Note 5**) coated Lab-Tek wells (approximately 500 μl of cell culture media in each well of a 8-well chamber slide) (MatTek dishes can also be used, *see* **Note 6**).
6. Incubate for 2–3 h (this is the time cells take to attach) before starting imaging.

3.1.3 Flow Cytometry Analysis

1. Resuspend 1–5 $\times 10^5$ dissociated mESCs in 4 % (v/v) FBS in PBS.
2. Gate live cells based on forward scatter and side scatter.
3. Use E14tg2a mESCs as a negative control to define fluorescence gates and determine fluorescence in NdC ESCs (*see* Fig. 1 for typical flow cytometry profiles) (*see* **Note 3**).

3.2 Time-Lapse Imaging

1. At least 2 hours before the experiments turn on the microscope incubator, with temperature set at 37 °C.
2. Half an hour before the experiment, turn on the lasers and the CO_2 controller.
3. Place a drop of immersion fluid (water or oil) on the objective.
4. Position the sample in the microscope and focus.
5. Select one area without cells to be used as background reference, to account for day-to-day laser's intensity fluctuations (*see* **Note 7**).
6. Select position(s) to be imaged. Select area(s) with "healthy" cells. If using multiple positions, select starting from left up and moving to the right down. The cell density in the imaging area should be chosen according to the duration of the experiment (*see* **Note 8**). For long acquisitions (>24 h), sparsely populated areas are a better choice. Typically, we select 20 positions for >24 h movie.

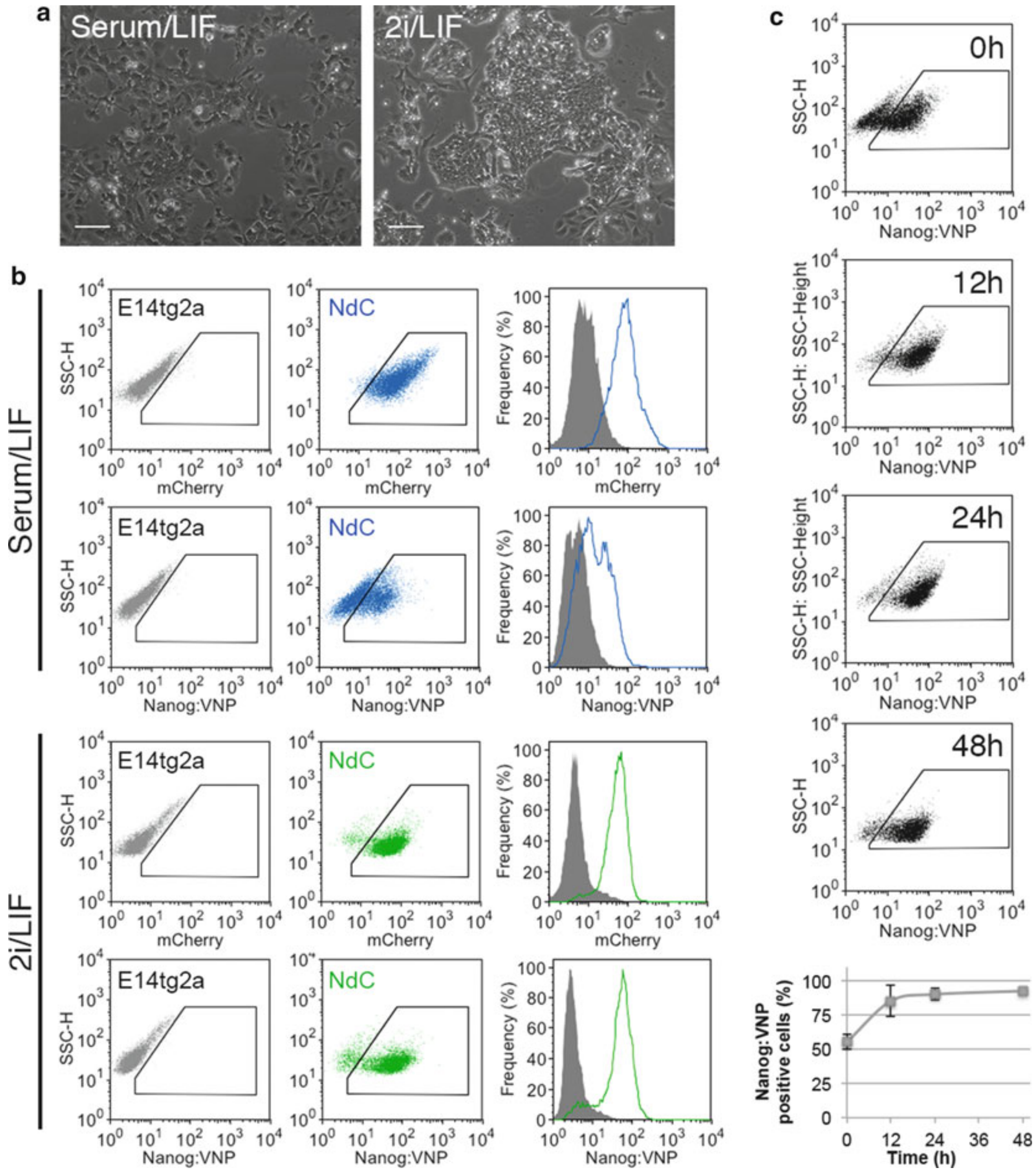

Fig. 1 Typical morphology and reporters flow cytometry profiles for NdC mESCs grown in Serum/LIF or 2i/LIF media. (**a**) Representative bright field images of NdC ESCs grown on poly-L-ornithine and laminin coated Lab-Tek wells. Cells were plated at a seeding density of 2 or 3 $\times$ 10^4 viable cells/cm^2, respectively for Serum/LIF or 2i/LIF media, grown at 37 °C incubator with a humidified atmosphere of 5 % CO_2 in air and images were taken after 36–48 h. Scale-bar: 100 μm. (**b**) Representative flow cytometry dot blots of Nanog:VNP and mCherry for E14tg2a (negative control, denoted in *gray*) and NdC cells (denoted in *blue* or *green*, respectively for Serum/LIF or 2i/LIF conditions). Positive gate regions were designed based on negative controls profiles. (**c**) Time course of VNP-positive cells, determined by flow cytometry, following transfer of NdC cells from serum/LIF to 2i/LIF medium

7. To allow for multiple positions measurement, record each position in the acquisition software.
8. If acquiring a z-stack set the lower and higher plane and the distance between planes (*see* **Note 9**).
9. Set the acquisition parameters: exposure time, time binning, and channels to be acquired. Contemporary acquisition of a phase contrast image is recommended.
10. Set imaging interval. Typical interval for short movies is around 2 min, while for longer movies images are acquired every 10/15 min.
11. Run a test acquisition.
12. Set the total experiment duration and begin acquisition.
13. It is advised to monitor the first half hour of acquisition to ensure that everything is running smoothly and, for long acquisitions, to regularly check on the status of the experiments.
14. At the end of the acquisition, before beginning data analysis, review the movies to check the quality of the acquisition.
15. Export movies. The final format will depend on the platform. In our case, each image in each channel was saved as a separate .tiff file, whose name contained information about the channel and the sequence. Different imaging platform might have different export options.

3.3 Tracking

Here we describe the procedure used in our lab to segment and track cells, based on a MATLAB software we have developed. The software is available upon request. It is also possible to perform the general steps of the process using different imaging software (e.g., ImageJ, Imaris).

1. Background subtraction. The software creates a mean image of the area without cells in each channel. This image is subtracted from all the subsequent images in each channel.
2. The software recreates a time series movie for each channel starting from the single .tiff images. (For other platform this step might not be necessary, as the files might be already exported as sequences.)
3. Segmentation—critical step. The software identifies each cell in a frame. Generally, it applies a threshold and morphological reconstruction to separate the fluorescent nucleus from the background. After segmentation, objects whose area is too big or too small to be a nucleus are removed. Segmentation is performed on the sequence of mCherry nuclear marker (Fig. 2a, c, d).
4. Inspect segmentation outcome and manually correct for mistakes.

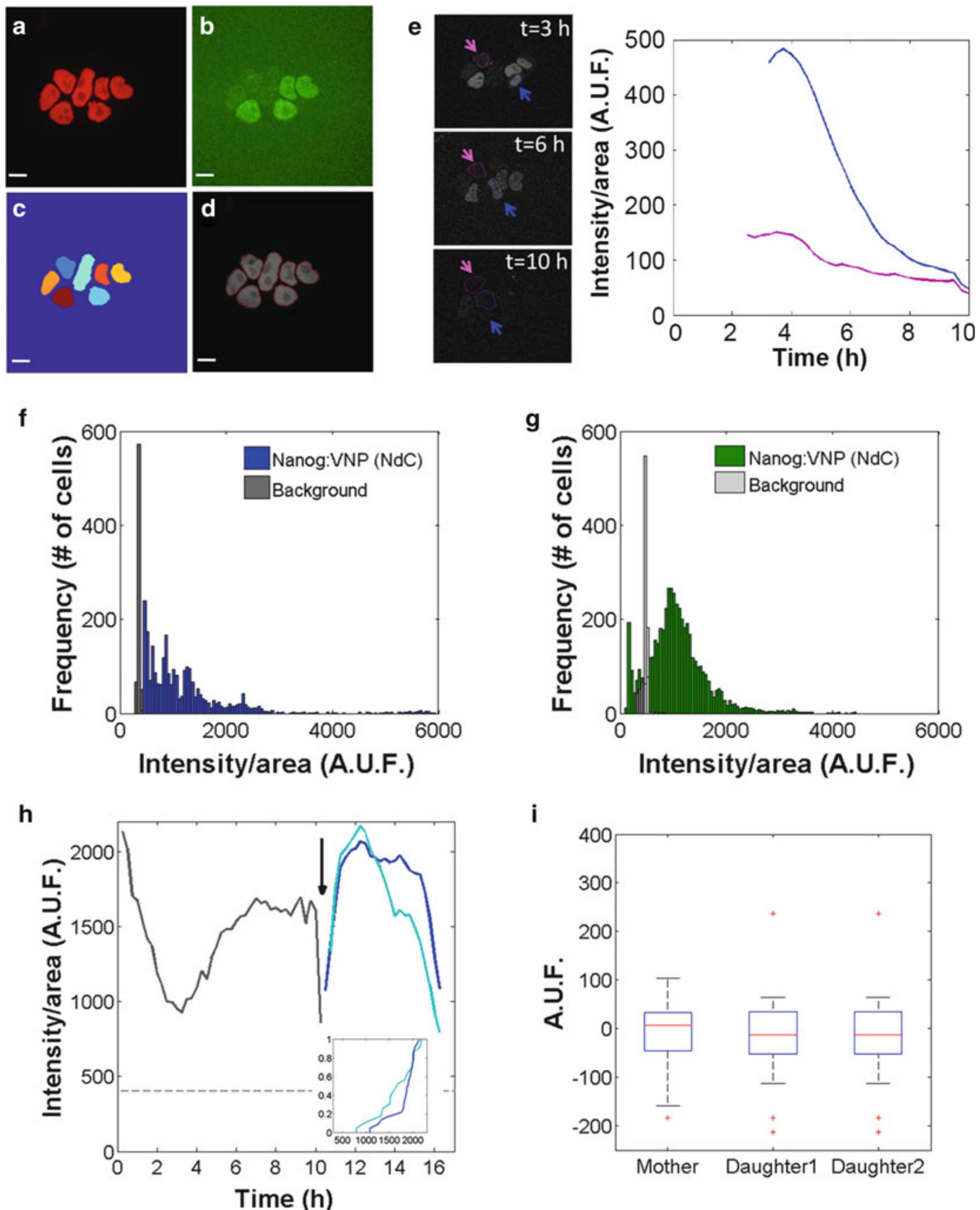

Fig. 2 Tracking of NdC mESCs and data analysis. (**a**) Representative mCherry nuclear stain of NdC cells grown in Serum/LIF. The image was colorized with ImageJ to aid visual recognition. (**b**) Representative Nanog:VNP nuclear stain of NdC cells shown in (**a**). The image was colorized with ImageJ to aid visual recognition. (**c**) Segmentation output, based on image in (**a**). Each cell is correctly identified and labeled according to a color code. (**d**) Nuclear perimeter of NdC cells, determined from (**b**) and overlaid to the original image (**a**). (**e**) Nanog:VNP fluorescence time trace of two NdC cells. (**f**) Histograms for Nanog:VNP fluorescence levels for NdC (*blue*) and E14tg2a (*gray*, negative control) cells grown in Serum/LIF media. All collected time points were

5. Labeling and tracking. Each cell is assigned a unique numerical label. Subsequently, tracking connects cells from consecutive images. To track cells between two frames, the overlap between two consequent frames is calculated. The process proceeds backwards, from the last frame to the first; thus, each cell division results in the disappearance of one cell.
6. Manually correct tracking. Each cell is represented with a color-coded nuclear contour (Movie 1, see Extras.springer.com) (*see* **Note 10**).

3.4 Data Analysis

A series of different parameters can be extracted from the movies. Here, we report a list of the most commonly used.

3.4.1 Fluorescence Intensity

1. Fluorescence distributions. The fluorescence intensity is calculated for each cell in each image and calculated values can be plotted as a histogram. This should be done for each culture media tested and background levels should be determined using ESCs with no fluorescent reporter (Fig. 2f, g, respectively for serum/LIF and 2i/LIF conditions) (*see* **Note 11**). To account for cell size discrepancies, the fluorescence intensity is usually normalized by the cell area. In the frames immediately before and after cell division, the cell spread out and fluorescence is lost. Therefore, these frames are usually discarded.
2. Fluorescence variation over time. Plot the fluorescence intensity calculated for each cell in each image vs. time. The intensity traces are retrieved for both channels (mCherry and VNP) (Fig. 2a, b). In Fig. 2e, the tracking of the VNP levels of two cells is illustrated.
3. Smoothing traces. When imaging with large time interval between frames (>5 min), it can be useful to apply a moving average smoothing to the intensity trace. Usually, three frames smoothing provides good results. The smoothing procedures allow to remove variation in intensity due to technical deviations (e.g., changes in focus or laser intensity).
4. Fluctuation Index. To compare the dynamic range of fluctuations in different conditions, the amplitude between the maximum and minimum fluorescence levels detected along an interphase can be calculated for each cell (Table 1).

Fig. 2 (continued) used. (**g**) Same as (**e**) for NdC (*green*) and E14tg2a (*gray*, negative control) cells grown in 2i/LIF media. (**h**) Nanog:VNP fluorescence time trace of a mother NdC cell and respective progeny, grown in Serum/LIF. *Arrow* indicates division time. *Inset plots* show the empirical cumulative distribution functions for each sister cell. (**i**) *Boxplot* representing the rates of gain and loss of fluorescence for the tracks shown in (**h**). Scale-bar for (**a**–**d**): 10 μm

Table 1
Cell cycle and fluctuation index parameters extracted from single cell time-lapse analysis of a Nanog reporter mESC line grown in two different cell culture media (Serum/LIF or 2i/LIF). Parameters relaying on fluorescence values (fluctuation index and rates) may vary depending on the microscope system used for cell tracking

Parameter	Serum/LIF	2i/LIF	References
Cell cycle (hours)	10 ± 2.3 ~12.6	12.3 ± 2.2 ~12.7	This chapter [12]
Fluctuation Index (A.U.F.)	486 ± 410 459 ± 448*	1033 ± 730 560 ± 311*	This chapter [12]

Note: these values were calculated considering only fluctuating cells

3.4.2 Cell Cycle Length

This is calculated as the time between two subsequent cell divisions (Table 1). Only cells for which both initial and final mitosis are observed should be considered.

3.4.3 Rate of Loss or Gain of Fluorescence

These parameters are estimated from the fluorescence vs. time tracks. To calculate the paces at which fluorescence changes occur, the rates of fluorescence increase and decrease (in arbitrary fluorescence units per time) for all cells are calculated from which mean values may be estimated. This data can also be plotted in the form of a histogram, where values around zero denote cells that show no fluctuations, and positive (gain rates) or negative (loss rates) values denote fluctuating cells (*see* **Note 12**).

3.4.4 Sister Cells Analysis

Using fluorescence vs. time tracks, the kinetics of sister cells can be analyzed (Fig. 2h). Different statistical tests can be used, such as the empirical cumulative distribution function (ecdf) and the Kolmogorov–Smirnov (K–S) test, to evaluate whether the curves belonging to sister cells come from the same distribution.

4 Notes

1. Always monitor cell morphology under an inverted microscope (typical cell morphologies are shown in Fig. 1) and passage cells when 70–80 % confluence is reached. If cells have not reached this after 48 h, keep changing the media every other day until the required confluency is achieved.
2. Upon culture media change, adaptation of mESCs is fast, and changes in NANOG expression are already detected after 12 h, stabilizing after 24 h (*see* Fig. 1c). These changes are accompanied by morphological alterations with the use of 2i/LIF media, resulting in more tightly packed mESCs colonies and a

reduction in flattened differentiated cells (10). Consequently, cell attachment in 2i/LIF conditions is poorer (especially on gelatin-coated dishes), and care should be taken not to wash away cell clusters during the passaging procedure.

3. When working with reporter cell lines, a routine check of the reporter expression is desirable. This can be quickly and quantitatively performed by flow cytometry analysis, from which the histogram of expression levels can be extracted.
4. The plating density may need to be optimized for each individual cell line, and for each cell culture media used. The chosen density should ensure that the lag phase of cell growth is minimized, and that confluency is only reached after 48 h. Typical plating densities for mESCs: 1–4 $\times$ 10^4 cells/cm^2. For longer time-lapse movies (more than 48 h), media change is required to ensure healthy cell maintenance.
5. mESCs are routinely grown on gelatin-coated polystyrene plates but for imaging purposes glass bottom plates have to be used. In these conditions, attachment is usually poorer and mESCs tend to grow even in more tightly compact colonies, hindering automated tracking of cells. The use of more efficient attachment substrates is therefore required and coating with poly-L-ornithing and laminin is a good alternative (fibronectin can also be used). Coatings should always be freshly prepared.
6. When Mattek dishes are used, resuspend cells in 400 μl media, add the mixture to the glass-covered portion of the dish, and incubate at 37 °C. After 2–3 h add 2 ml of preheated media at 37 °C. Incubate for 1–2 h at 37 °C and proceed to imaging. Equilibration times at 37 °C are critical to avoid cell detachment upon transfer for the microscope settings.
7. Laser intensity and illumination time should be previously determined, since they depend on the chosen fluorophore and the duration of the experiment. Ideally, these parameters should be optimized to minimize photobleaching and cell death, while allowing for high image quality. We suggest running a series of test experiments to see which values fit best the cell sample. Once the parameters have been chosen, they should be kept the same for all the experiments to allow for comparison.
8. When planning to monitor for longer time periods, it is better to choose areas with lower cell density for imaging. If cells grow in layers it becomes difficult or not possible at all to track them automatically or even manually.
9. When performing time-lapse movies, it is recommended to acquire a z-stack, by setting the lower and higher plane and the distance between planes. This will prevent loss of cells due to cell movement across planes, which is likely to occur during cell division.

10. The software offers the opportunity to choose between removing or keeping cells on the border of the image. Removing cells on the border reduces the number of incomplete tracks (a cell that goes in and out of the imaging area will be counted as a new one at each reappearance).
11. Different media result in different fluorescent backgrounds. Thus, for every media used, it is necessary to acquire a set of images of nonfluorescent cells (at each chosen wavelength) to determine the background level of fluorescence. This is necessary especially when comparing results from different conditions. On top of this control, day-to-day variation in laser intensity might cause slight changes in background fluorescence. Thus, it is important to acquire a movie of an area without cells to use as a background reference.
12. Fluctuation rates are calculated as relative values and are independent of the absolute number of reporter molecules per cell. Consequently, two individual mESCs can show similar fluctuation rates while having different reporter protein concentrations.

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Electronic Supplementary Material

Below is the link to the electronic supplementary material.Movie 1Representative time-lapse movie where each cell is represented with a color-coded nuclear contour (MOV 872 kb)

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Expanding the Utility of FUCCI Reporters Using FACS-Based Omics Analysis

James Chappell, Ben Boward, and Stephen Dalton

Abstract

The FUCCI indicator system is a powerful tool for spatio-temporal analysis of the cell cycle, but its utility has been restricted so far to a limited range of applications. Here, we describe how to establish and validate the FUCCI system in murine pluripotent stem cells (PSCs) and describe the utility of transgenic FUCCI mice. We then describe how the FUCCI system can be used to generate material for a wide-range of omics-based applications in conjunction with FACS isolation of cells. This significantly broadens the potential applications of FUCCI reporters for studying the molecular basis of development and disease.

Keywords: FUCCI, Cell cycle, Stem cells, Pluripotency, FACS

1 Introduction

The "fluorescent ubiquitination-based cell cycle indicator" (FUCCI) system is a valuable tool for characterizing spatio-temporal regulation of cell division (1–5). Sakaue-Sawano and colleagues genetically engineered two fusion proteins, monomeric Kusabira Orange with a Cdt1 truncation mutant (mKO2-Cdt1) and monomeric Azami Green with a Geminin truncation mutant (mAG-Gem), which oscillate via posttranslational control in order to indicate individual cell cycle phases with a unique fluorescent signature (4). This system has proven to be a dramatic improvement compared to previous methodologies for interrogating cell cycle events in mammalian cells. Previously, the field relied heavily on pharmacological treatment to synchronize cells but this is problematic due to cytotoxicity and because cell cycle aberrations are frequently observed under these conditions.

Two recent reports have coupled the FUCCI indicator system to fluorescence-activated cell sorting (FACS) to expand the utility of this system to include molecular approaches such as qRT-PCR, western blots, chromatin immunoprecipitation (ChIP) (6, 7) and at the omics levels, RNA-seq (6). In these two cases, cultured pluripotent stem cells (PSCs) were used as a biological platform. In this report, we describe in detail how to establish the FUCCI system in

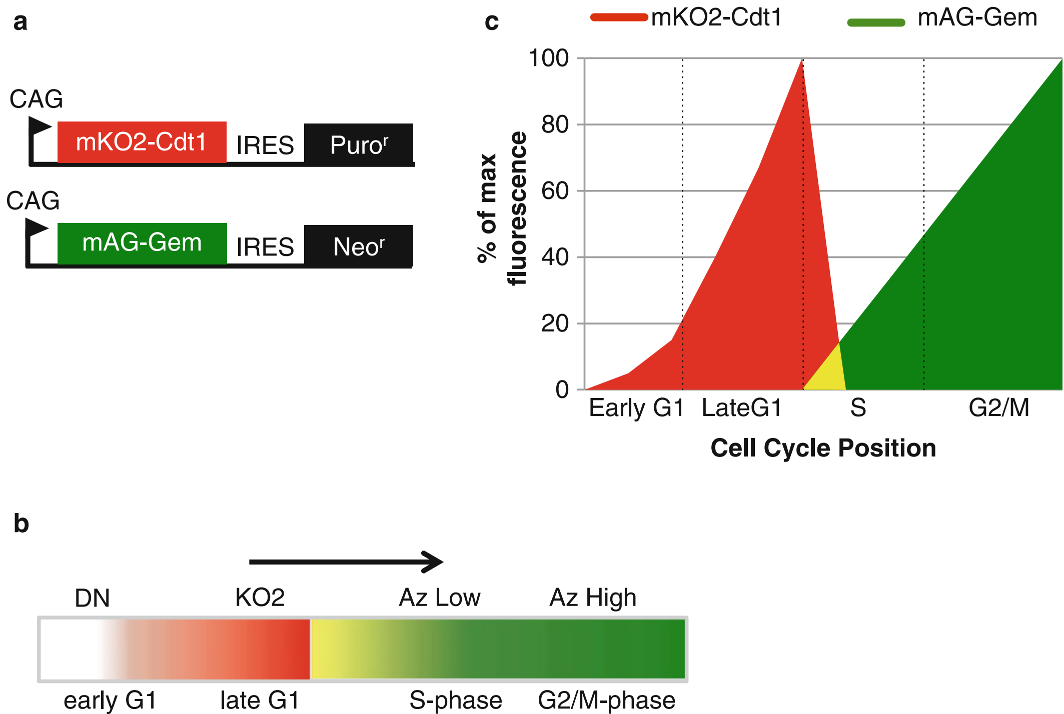

Fig. 1 (**a**) Diagram of FUCCI reporters (**b**) Schematic linking fluorescent protein expression patterns with cell cycle phase. (**c**) Oscillations of FUCCI reporter fluorescence during cell cycle progression

PSCs and how to interpret the fluorescent signatures of cells (Fig. 1). We then describe how to couple the FUCCI system with FACS isolation of cells in a distinct cell cycle phase from a heterogeneous population of PSCs and from tissues of transgenic mice (Fig. 2). Lastly, we demonstrate how implementation of this strategy followed by focused molecular analyses, such as transcript and protein expression analysis, and omics-based analyses, such as proteomics, ChIP-seq, chromatin conformation capture assays (3C, 4C, 5C, HiC, ChIA-PET) (8), and epigenomics (DNA methylation) enhances the ability to characterize the molecular mechanisms involved in development and disease (Fig. 3).

2 Materials

The following materials are required for establishment, characterization, and FACS of FUCCI PSCs and primary cells.

1. FUCCI DNA Vectors (http://www.mblintl.com/search/product-results.aspx?k=Fucci&submitsearch=%C2%A0&a=0&c=0&co=0&h=0&p=0&s=0&st=0&ct=0).

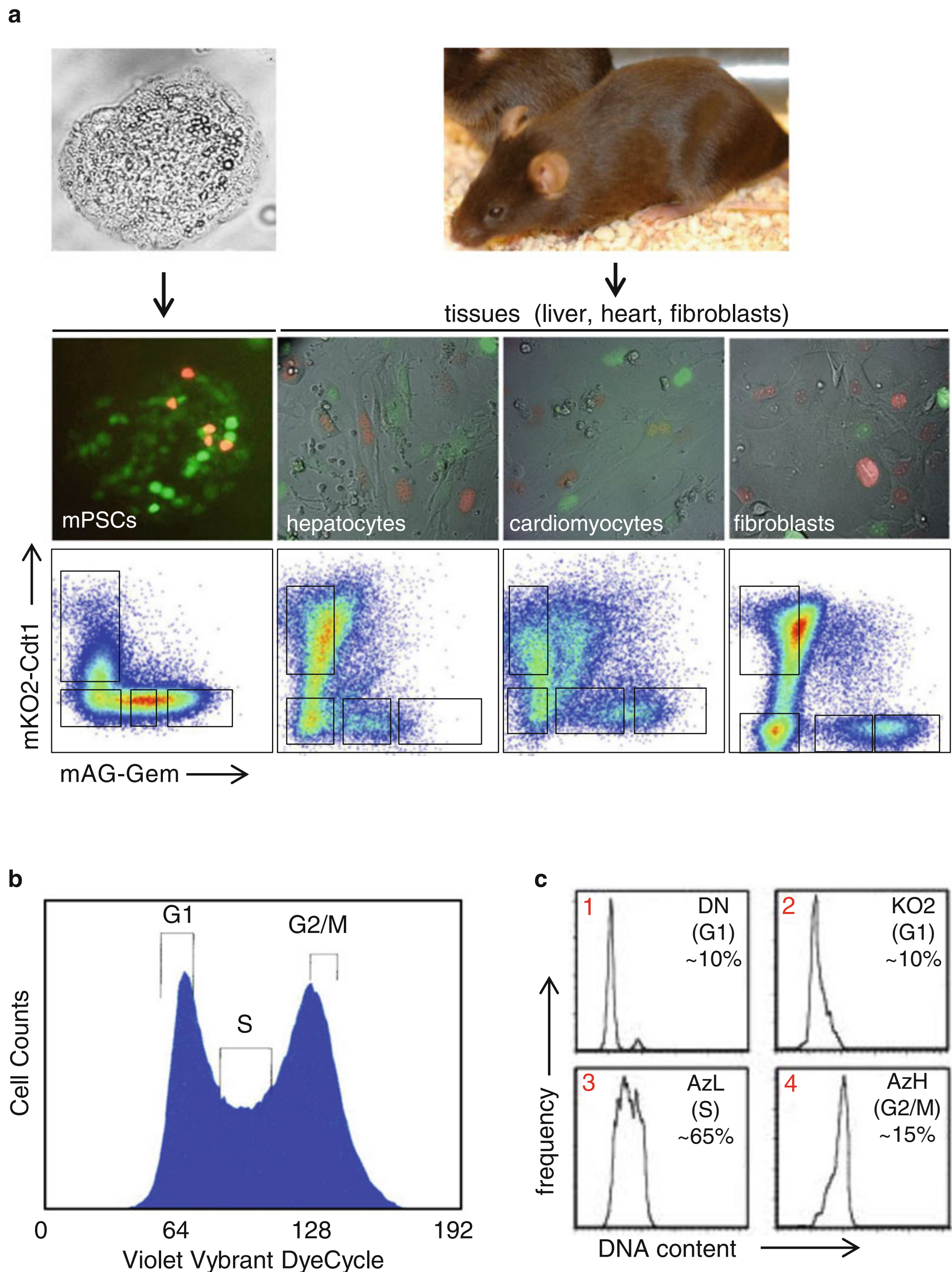

Fig. 2 (**a**) Murine PSCs and primary cells from FUCCI mice can be isolated and used for imaging or isolation by FACS. *Boxes* on FACS profiles indicate gating; *see* Table 2 for the number of cells collected per gate. (**b**) FUCCI mPSCs stained with Violet Vybrant DyeCycle and analyzed for DNA content by flow cytometry. (**c**) Cell cycle fractions following FACS, stained with Violet Vybrant DyeCycle and reanalyzed for DNA content

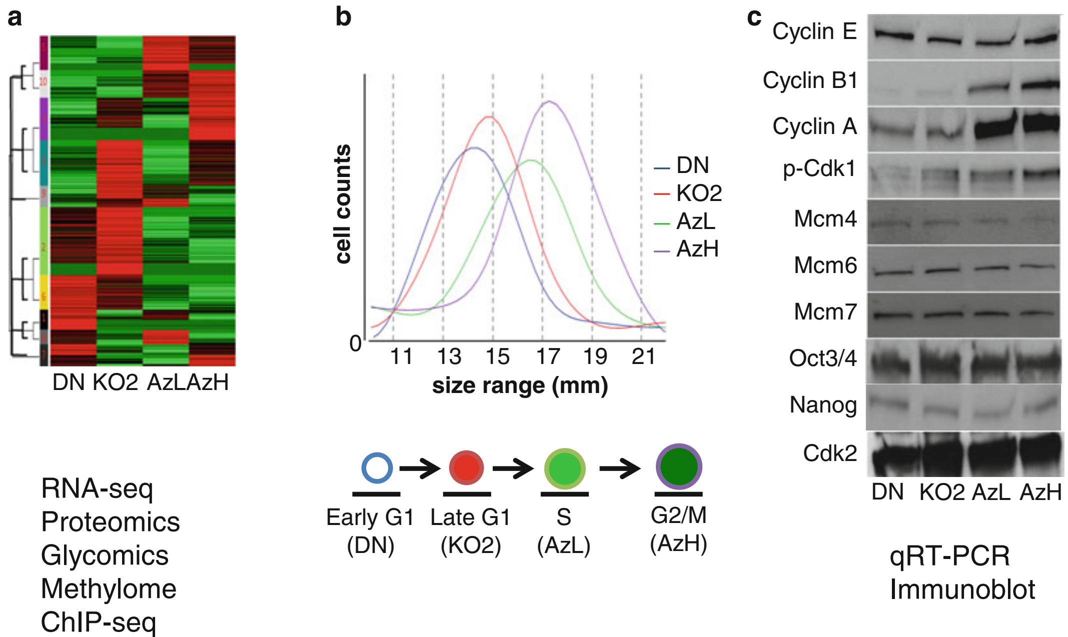

Fig. 3 Example analyses that can be performed following FACS. (**a**) Omics, (**b**) cell size analysis, (**c**) focused analysis

2. FUCCI mice: (http://www.amalgaam.co.jp/products/advanced/fucci.html).
3. Mouse PSC culture medium: fetal calf serum (10 %), KnockOut serum replacement (10 %) (Life Technologies #10828-028), Leukemia Inhibitory Factor (1,000 units/mL) (Millipore #ESG1107), Sodium Pyruvate (100 mM), L-glutamine (200 mM), Penicillin-Streptomycin solution (500 μg/mL), β-mercaptoethanol (0.1 mM). Combine these components into a base of Dulbecco's Modified Eagle Medium (DMEM) and run through a filter flask with 0.45 μm pore size.
4. Drugs for selection of FUCCI markers (Puromycin and Neomycin recommended for mPSCs).
5. Vybrant DyeCycle Violet (Life Technologies #V35003).
6. Dulbecco's Phosphate-Buffered Saline (DPBS; 1×).
7. 5 mL cell strainer tubes.
8. 5 mL round-bottom collection tubes.
9. Accutase (Life Technologies #A1110501).
10. Anti-Kusabira Orange 2 (MBL International #M168-3).
11. Anti-monomeric Azami-Green 1 (MBL International #PM052).

3 Methods

3.1 Establishment of the FUCCI System in PSC Lines

Investigating potential connections between the cell cycle machinery and cell fate decisions has been a difficult task due to a lack of suitable tools. Previous studies relied on small molecules, such as aphidicolin and nocodazole, to synchronize cells in a particular cell cycle phase, with the unintended complications of acute cytotoxicity, metabolic perturbations, and a diminished ability for cell cycle reentry (9). Implementation of the FUCCI system alleviates these complications and permits interrogation of cell cycle–controlled mechanisms in PSCs and their derivatives.

1. Subclone the mKO2-Cdt1 DNA fragment into an appropriate expression plasmid vector (*see* **Note 1**) (Fig. 1a).
2. Transfect the mKO2-Cdt1 expression plasmid into cells.
3. Apply drug selection until untransfected cells die off (*see* **Note 2**).
4. Establish a stable cell line by isolating and clonally expanding cells that display mKO2 fluorescence (*see* **Note 3**).
5. Subclone the mAG-Gem DNA fragment into an appropriate expression plasmid vector (Fig. 1a).
6. Transfect the mAG-Gem expression plasmid into cells from **step 4**.
7. Establish a stable cell line by isolating and clonally expanding cells that display mAG fluorescence (*see* **Note 4**).
8. Confirm that colonies contain cells expressing FUCCI markers under a microscope (Fig. 3c, *see* **Note 5**).

3.2 Confirmation of FUCCI Expression in Mice

Transgenic FUCCI mice (*see* Section 2) allow researchers to study aspects of cell cycle control as it relates to disease, tissue repair, regeneration, and aging. While maintaining colonies is straightforward, we have noted that they exhibit reduced fecundity in comparison to other wild-type strains, as evidenced by small litter sizes. We have found it advantageous to outcross with a Swiss Webster mouse prior to experiments requiring large tissue numbers. In this situation, the following steps should be taken to screen for proper FUCCI marker expression in the offspring prior to experimentation.

1. Cut off a small (~1 mm) piece from the tail tip of a neonatal FUCCI mouse.
2. Place the section on a microscope slide in a drop of DPBS and add a coverslip.
3. Confirm positive mKO2 expression by observing under a fluorescence microscope using a green laser (488 nm).
4. Confirm positive mAG expression by observing under a fluorescence microscope using a red laser (561 nm).

3.3 FACS Sorting of FUCCI Cells

The FUCCI system has proven to be a valuable tool for characterizing spatio-temporal aspects of cell division. By combining FUCCI fluorescence with FACS isolation of cell cycle fractions, it is now feasible to perform downstream analyses to better investigate hypotheses regarding molecular aspects of cell cycle control. Furthermore, using this highly reproducible protocol makes it possible to combine equivalent sorted fractions from multiple FACS isolations, allowing for large scale analyses, such as chromatin conformation capture assays (3C, 4C, 5C, HiC, ChIA-PET) (8) that require tens of millions of cells.

1. Determine an appropriate number of PSCs, or mice, required for intended analysis (*see* **Note 6**, Tables 1 and 2).
2. Create a single-cell suspension from mPSCs, or isolated tissues of interest (*see* **Note 7**).
3. Adjust the cell suspension concentration to 10,000,000 cells/mL.

Table 1
Example cell numbers needed for a variety of downstream analyses

Experiment	Cell # needed
RNA-seq[a]	$1–10^5$
qRT-PCR[a]	$1–10^5$
Western Blot	5×10^5
Proteomics	$5 \times 10^6–10^7$
ChIP	$10^3–10^6$
3C, 4C, 5C, HiC, ChIA-PET	$5 \times 10^7–1 \times 10^8$

[a]Single cell analysis is available (13) but currently uncommon. Upper estimates are reflective of current standard technologies

Table 2
Approximate cell numbers from one confluent 10 cm culture dish[a], or one tissue[b], and expected cell numbers for each cell cycle fraction following FACS from a starting population of one million cells

Cell type	Cell #	Early G1 (%)	Late G1 (%)	S (%)	G2/M (%)
[a]mPSC	[a]2×10^7	2×10^6 (10 %)	1×10^6 (5 %)	2×10^6 (10 %)	2×10^6 (10 %)
[b]Cardiomyocytes	[b]2×10^6	2×10^5 (10 %)	3×10^5 (15 %)	1×10^5 (5 %)	1×10^5 (5 %)
[b]Hepatocytes	[b]2×10^6	2×10^5 (10 %)	6×10^5 (30 %)	1×10^5 (5 %)	6×10^4 (3 %)
[a]Tail tip fibroblasts	[a]1×10^7	1.5×10^6 (15 %)	6×10^6 (60 %)	5×10^5 (5 %)	5×10^5 (5 %)

4. Filter the cell suspension using cell strainer tubes appropriate for the FACS apparatus.
5. Initialize the flow cytometer by setting the 488 and 561 laser outputs to 100 mW and setting the voltage of the mKO2 detector to 406 and the mAG detector to 325 (*see* **Note 8**).
6. Run approximately 50,000–100,000 cells at a rate of 10,000 events per second to set gating.
7. Gate out dead cells and debris by forward scatter versus side scatter gating (*see* **Note 9**).
8. Gate cells from **step 8** by mKO2 versus mAG for the cell cycle fractions of interest (*see* **Note 10**) (Fig. 3c, d).
9. Sort cells into FACS collection tubes containing 200 μL of culture medium, which will increase post-FACS viability.

3.4 Validation of Sorted Fractions by DNA Content

Different cell types will show distinct patterns of FUCCI marker expression, reflecting differences in rates of cell cycle progression (Fig. 2a). This can complicate FACS and downstream analysis. Therefore, it is necessary to validate the sorted fractions using a DNA content stain and optimize the protocol for the cell type of interest.

1. Dilute FACS sorted cell cycle fractions and unsorted cells to a concentration of 10^6 cells/mL in 1× DPBS.
2. Aliquot 1 mL to a 5 mL polystyrene round-bottom FACS tube.
3. Add 1 μL of Vybrant DyeCycle Violet to the tube (*see* **Note 11**).
4. Incubate for 30 min at 37 °C in the dark (*see* **Note 12**).
5. Place on ice.
6. Analyze DNA content of viable cells on a flow cytometer (*see* Fig. 2b and **Note 13**).
7. Plot cell count frequency versus DNA content to determine the purity of the FACS sort (*see* Fig. 2c and **Note 14**).

3.5 Conclusion

The FUCCI system has previously allowed researchers to characterize spatio-temporal aspects of cell division. The methods described here for establishing FUCCI PSC lines and primary cells from transgenic mice, coupled with the use of FACS, increase the capacity to perform a variety of omics-type analyses that will reveal the cell cycle–specific molecular mechanisms important for disease and development.

4 Notes

1. An appropriate plasmid expression vector should contain a promoter that drives constitutive expression of the DNA fragment; CAGi works well in R1 mPSCs (Fig. 3a). Also, each of

the two FUCCI reporters should be cloned into vectors containing drug selectable markers. Puromycin and neomycin selection are recommended using expression cassettes linking them to the fluorescent protein reporter component via an internal ribosome entry site (IRES). For R1 mPSCs, we recommend using puromycin at a concentration of 1 μg/mL and neomycin at a concentration of 200 μg/mL.

2. The time needed for drug selection to complete varies based on the selectable marker used. Puromycin usually kills untransfected cells within a few days and transfected cells will amplify after approximately 1 week. Neomycin can take slightly longer.
3. Single mKO2 positive cells can be isolated via FACS, or colonies can be picked under a fluorescent scope. It is unlikely that an entire colony will be mKO2 positive, as approximately 5–10 % of mPSCs are in late G1 at any one time.
4. Cells successfully transfected with mAG-Gem can display a large range of mAG expression: G1 cells will not display mAG, S-phase cells will display low levels of mAG, and G2/M cells will display high levels of mAG.
5. Expression of FUCCI proteins can be further validated by immunofluorescent staining and/or western blot analysis using commercially available antibodies (*see* Section 2).
6. We established the FUCCI system in R1 murine ES cells. We seeded 0.5×10^6 cells per 10 cm dish, and then incubated for 3 days at 37 °C with 5 % CO_2 to obtain a confluent culture. Example tissues demonstrated here were isolated and cultured as previously described: tail tip fibroblasts (10), neonatal cardiomyocytes (11), fetal hepatocytes (12). The number of cells, or tissues, needed to perform a given experiment will vary (*see* Table 1), and may require combining equivalent fractions from multiple sorts. Furthermore, single cell analysis of transcript expression is available (13) but currently uncommon.
7. While trypsin (0.05 %)-EDTA is often used to create single cell suspensions of mPSCs, we have found that Accutase is superior for reducing cell clumping that would otherwise disrupt the flow cytometer.
8. Parameters specified herein are for use with the Beckman Coulter MoFlo XDP cell sorter and will likely require customization for other sorters. In this setup, mKO2 fluorescence was excited by a 561 nm laser and emission detected with a 586/30 nm bandpass filter. mAG fluorescence was excited by a 488 nm laser and emission detected with a 529/28 nm bandpass filter. Both fluorescent protein emissions were measured in log and compensation was performed as needed to subtract overlapping emissions.

9. Forward scatter and side scatter readings were collected in linear mode off the 488 nm laser excitation. Usually two distinct populations are evident, where the cells appearing nearest to 0 on the *X*- and *Y*-axis are either dead cells or debris and the larger population represents viable cells.
10. Cells that are double negative (DN) for mKO2 and mAG are in the early G1 phase. Cells positive for mKO2 and negative for mAG (KO2) are in late G1 phase. Cells negative for mKO2 and with moderate mAG expression (AzL) are in S phase. Cells negative for mKO2 and highly positive for mAG (AzH) are in G2/M phase. Gating different percentages of each fraction will affect the purity and exact cell cycle phase composition of the sorted cells. The percentage of cells in each cell cycle phase can vary greatly among different cell types (*see* Table 2, Fig. 2a). For this reason, combining equivalent fractions from multiple sorts may be necessary for some downstream analyses.
11. The amount of DyeCycle reagent to be added here may need to be optimized for specific cell lines. mPSCs have been known to efflux dyes, and therefore, an increased amount may need to be used if insufficient staining is observed.
12. The incubation time with the dye can also be fine-tuned in order to optimize staining.
13. In this setup, forward scatter and side scatter were collected in linear mode off the 488 nm laser excitation and used to exclude dead cells and debris. DyeCycle Violet fluorescence was excited by 405 nm laser excitation (Coherent) and detected using a 447/55 bandpass emission filter.
14. The percentage of cells in cell cycle phases can be analyzed using the FlowJo Cell Cycle platform (http://www.flowjo.com/).

Acknowledgements

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Selection of Antibodies Interfering with Cell Surface Receptor Signaling Using Embryonic Stem Cell Differentiation

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Abstract

Antibodies able to bind and modify the function of cell surface signaling components in vivo are increasingly being used as therapeutic drugs. The identification of such "functional" antibodies from within large antibody pools is, therefore, the subject of intense research. Here we describe a novel cell-based expression and reporting system for the identification of functional antibodies from antigen-binding populations preselected with phage display. The system involves inducible expression of the antibody gene population from the *Rosa-26* locus of embryonic stem (ES) cells, followed by secretion of the antibodies during ES cell differentiation. Target antigens are cell–surface signaling components (receptors or ligands) with a known effect on the direction of cell differentiation (FGFR1 mediating ES cell exit from self renewal in this particular protocol). Therefore, inhibition or activation of these components by functional antibodies in a few elite clones causes a shift in the differentiation outcomes of these clones, leading to their phenotypic selection. Functional antibody genes are then recovered from positive clones and used to produce the purified antibodies, which can be tested for their ability to affect cell fates exogenously. Identified functional antibody genes can be further introduced in different stem cell types. Inducible expression of functional antibodies has a temporally controlled protein-knockdown capability, which can be used to study the unknown role of the signaling pathway in different developmental contexts. Moreover, it provides a means for control of stem cell differentiation with potential in vivo applications.

Keywords: Phage display, FGF4, FGFR1, ES cell differentiation, Phenotypic selection, Protein knockdown, Functional antibody, Cell surface receptor signaling

1 Introduction

Antibodies that modify cell signaling by binding to ligands or receptors have proven value as drugs for a diversity of conditions and the demand for such therapeutic antibodies continues to grow [1, 2]. With their exquisite specificity, functional antibodies have also unexploited potential in the control of stem cell fate specification. Phage- or yeast-display antibody libraries provide a source of rich diversity and can generate hundreds of antibodies to a single target antigen [3, 4]. However, screening for functional antibodies, which modify cellular function, from such large libraries can be quite tedious and time consuming. The process involves screening

of large numbers of candidate antibodies by ELISA against the target antigen, followed by expression and purification of binding antibodies, which then have to be tested on target-specific reporter cell and in vitro assays. It also requires bacterial production and purification of antibodies, which has the risk of compromising reporter cell response with endotoxins. Alternatively the antibodies may have to be individually cloned into mammalian expression vectors before expression and purification from mammalian cells.

In this chapter we describe a novel cell-based screen for functional antibodies based on their ability to inhibit or activate cell surface signaling components, which mediate differentiation of embryonic stem (ES) cells. ES cells are derived from the inner cell mass of the blastocyst-stage embryo and they retain their pluripotent character in vitro, i.e., they are able to differentiate to many different cell types [5]. We first validated and exemplified this method in Melidoni et al. [6] by generating antibodies that block FGF4 signaling through inhibition of either ligand or receptor. Antibody populations against the signaling component of interest are preselected for their ability to bind the target antigen from a phage-display library [4]. Binding antibody populations are inducibly expressed and secreted during ES cell differentiation, followed by phenotypic selection of clones exhibiting altered differentiation outcomes, as a result of functional antibodies interfering with cell surface receptor signaling. We call this *ES* cell-based *I*n-*C*ell *E*xpression and *R*eporting (*ES-ICER*) system. The basic steps of the method are the following (*see* Fig. 1A): The target antigen-binding antibody population is cloned into a suitable targeting vector and introduced into the ubiquitously expressed *Rosa-26* [7] locus of an appropriate ES cell reporter line by homologous recombination. This process leads to the introduction of an average of one antibody per ES cell. Positive selection by a drug selection marker follows and the selected ES cell clones are subject to differentiation, during which the expression and secretion of the antibodies is temporally regulated with the use of an inducible promoter (such as the Tet-On system). Functional antibodies secreted around a few elite ES cell clones inhibit or activate the target signaling component, resulting in a shift in the relevant differentiation outcome of the cells, as monitored by expression of the (usually fluorescent) reporter marker. Therefore, the choice of a good differentiation assay in conjunction with a suitable reporter ES cell line is essential to enable the phenotypic selection of potent antibodies with the ES-ICER system. Physical separation of the ES clones between them by various means at the differentiation stage ensures the functional effect of the few elite antibodies is not diluted by the nonfunctional majority. Consequently, ES cell colonies, each encoding a unique functional antibody (*ES-ICER clones*), are identified. Functional antibody genes are recovered from these colonies by PCR, transiently expressed in mammalian cells (such as Human

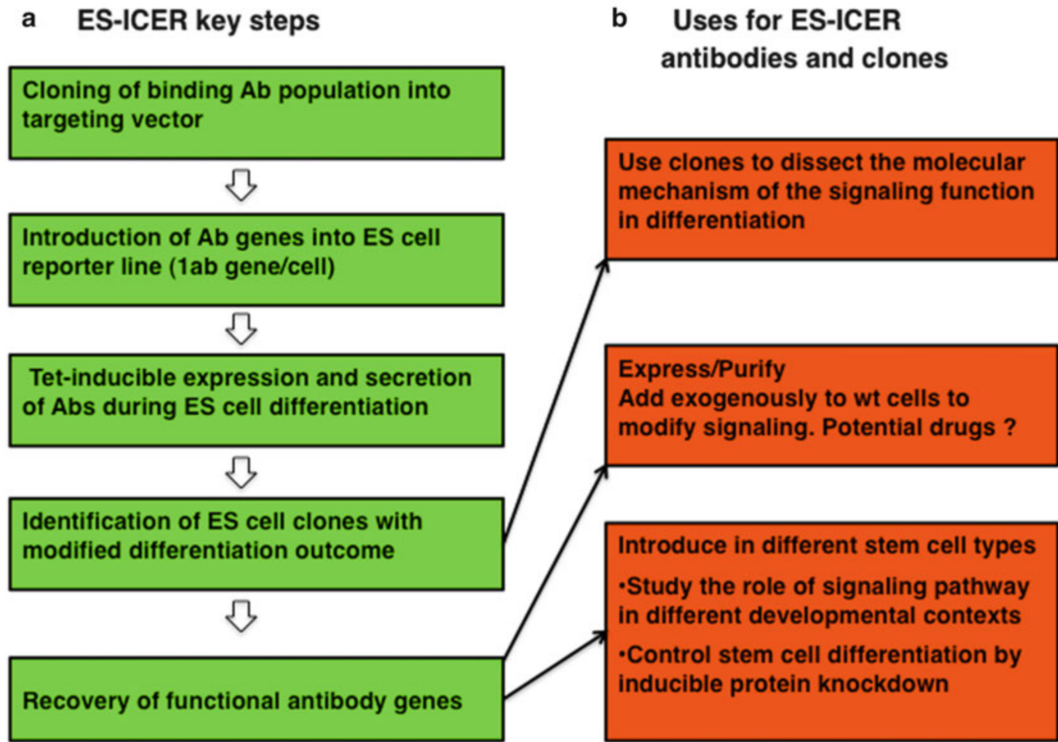

Fig. 1 (*A*) The key steps of the ES-ICER method. (*B*) Different uses of the functional antibodies and functional antibody-expressing ES cell clones derived from ES-ICER

Embryonic Kidney 293) and purified. In order to confirm activity, purified antibodies are added exogenously to wt cells and tested for their ability to modify the signaling component's effect on the differentiation of the cells.

During ES cell differentiation stem and progenitor cell fates are influenced by a plethora of cell surface receptors conveying signaling from the environment [8, 9]. This creates the potential to target receptors as diverse as RTKs, GPCRs, ion channels and cell adhesion molecules, and ligands, such as growth factors, peptides, cytokines and hormones, assuming their effect in cell specification is defined for a particular cell type at a specific differentiation stage Therefore, the power of the ES-ICER system is that it is intrinsically versatile, potentially allowing the identification of functional antibodies in a variety of developmental contexts (*see* Fig. 2).

In this protocol, a detailed method is described of the differentiation assay designed to identify functional antibodies inhibiting FGF4-mediated signaling, through inhibition of the initial stage of differentiation of mouse ES cells (*see* Section 3.6). Important aspects of the differentiation assay design are also highlighted in Section 3.5).

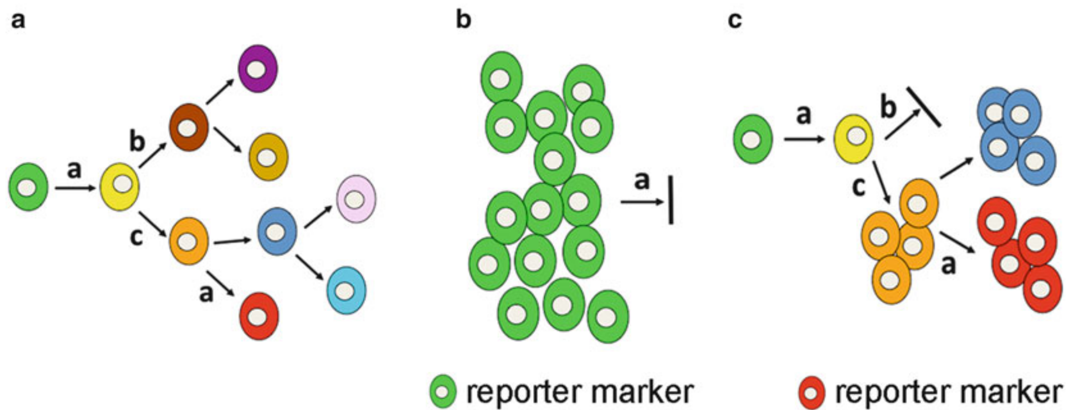

Fig. 2 The versatility of the ES-ICER system allows the identification of functional antibodies that affect cell surface signaling-mediated cell fates in a variety of developmental contexts. Pluripotent ES cells are able to differentiate in vitro to multiple cell lineages the creation of which is regulated by different signaling pathways operating at different stages. (**a**) is a schematic representation of this process with "a", "b", and "c" representing the different signaling pathways, while the different cell lineages are depicted with different colors. Blocking a specific pathway at a specific time point with a functional antibody under differentiation conditions of interest, one can modify the differentiation output and detect this with molecular readouts and engineered reporter cell lines. For example, by blocking signaling pathway "a" at the initial differentiation stage, we might observe a proliferation of the (*green*) ES cell population, (**b**), or inhibition of pathway "b" could lead to an accumulation of "*red*" and/or "*blue*" cells that we can detect and quantitate

The versatility of the ES-ICER system extends to the potential uses of its final products, the functional antibodies and the functional antibody-encoding ES cell clones (Fig. 1B). As mentioned above, the purified functional antibody can be added exogenously to affect cell signaling in any cell of interest. An obvious implementation of this is in the control of fate specification of human stem cells or stem-cell like cells, such as cancer stem cells in vivo. On the other hand, the antibody-encoding ES cell clones constitute useful tools by which to analyze the molecular mechanism of the interrogated signaling pathway's function at the given cell differentiation stage. Furthermore, once a functional antibody has been identified by the ES-ICER method, its gene can be introduced into a different reporter ES cell line, serving a dual role this time: (a) as a tool for the investigation of the unknown role of the signaling pathway in different developmental contexts and (b) as a tool to control stem cell differentiation (*see also* Fig. 3). These clones, encoding an inducible functional antibody, have a protein-knockdown capability and, therefore, unlike inducible RNAi they permit exquisite temporal control of stable signaling components at the protein level.

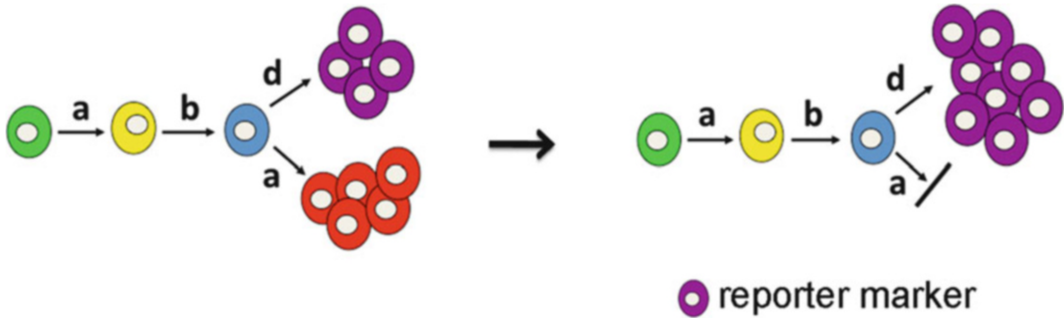

Fig. 3 The ES-ICER system as a means of inducible protein knockdown to control stem cell differentiation. In this hypothetical example, an antibody that was shown in Fig. 2b to block the activity of signaling pathway "a" at an early differentiation stage, causing the accumulation of cells carrying the "*green*" reporter, is used here to inhibit signaling pathway "a" at a later differentiation stage. For this purpose, the antibody gene is introduced into a different reporter cell line; its expression induced at a different time point in a different differentiation assay. Antibody activity blocks the generation of cells carrying the "*red*" cells, shifting the differentiation towards the "*purple*" output that can be detected either with a second reporter marker or with immunostaining. Hence, combined with a good differentiation protocol, the timely expression of an appropriate antibody can promote directed differentiation and production of pure cellular populations with potential therapeutic applications

2 Materials

2.1 Antibody Populations for In-Cell Expression in ES Cells

Antibody populations for ES-ICER expression could be derived from display methods such as phage display, yeast display, or ribosome display [10]. Such populations may be derived from large, naïve display libraries, where pools of antibodies, specific to a range of antigen epitopes, may be generated by selection against purified antigen as previously described [4]. Alternatively antibody populations could potentially be generated from PCR amplification of V gene populations from the B cells of immunized animals. Potential sources of antibody phage-display libraries are Creative Biolabs (http://www.creative-biolabs.com) or Source Bioscience (http://www.lifesciences.sourcebioscience.com/products/antibodies/proteomics/human-domain-antibody-library/?rd=1). The protocol described herein is based on the use of an antibody population, in the form of single-chain Fvs (sc-Fvs) derived from the "McCafferty phage-display library" [4].

2.2 Materials for Cloning of Antibody Genes into pBIOCAM5-GW Entry Vector

1. Vector harboring the selected sc-Fvs populations as described in Section 2.1 (e.g., pSANG4 phagemid vector, Schofield et al.). Glycerol stocks are stored at −80 °C.
2. pBIOCAM5-GW targeting vector available from Addgene (https://www.addgene.org/John_McCafferty/).
3. HotStar Taq DNA polymerase, buffer, dNTPs (2 mM) and $MgSO_4$ (25 mM) are available as part of a kit from Qiagen (203203). Store at −20 °C.

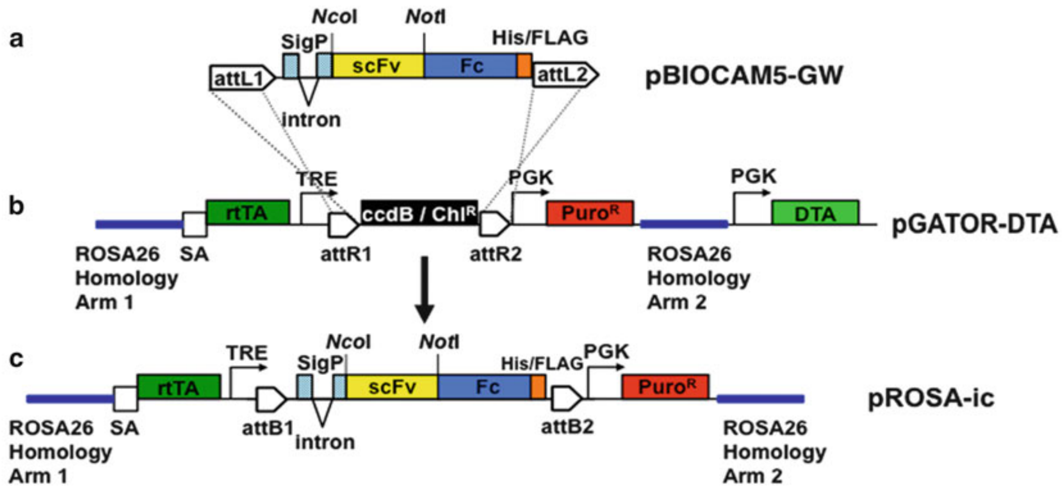

Fig. 4 Construction of the targeting vector pROSA-ic. (**a**) The plasmid pBIOCAM5-GW, encoding the pre-selected antibody population genes in the form of single-chain Fv (scFv-Fc fusions). pBIOCAM5-GW antibody library is GATEWAY LR recombined [19] with the plasmid pGATOR-DTA (**b**) to give the plasmid pROSA-ic (**c**). The diphtheria toxin gene (DTA) in pGATOR-DTA is used to reduce random integration of the cassette in the genome. The final construct contains a "Tet-On" transcriptional system for doxycycline-dependent antibody expression during ES cell differentiation. A signal peptide directs secretion of the resulting scFv-Fc fusion and a His-FLAG tags the C-terminus of the scFv-Fc fusion genes. Abbreviations include: *attL1*, *attL2*, *attR1*, *attR2*, *attB1*, *attB2* gateway recombination sites; *sigP* signal peptide; *His/FLAG* hexa-histidine Tri-FLAG peptide tag; *scFv* single-chain Fv; *Fc* human IgG1 Fc; *SA* splice acceptor; *rtTA* Tet-On transactivator; *TRE* Tet response element; *PuroR* puromycin resistance gene; *DTA* diphtheria toxin gene fragment A. This figure has been adapted from Melidoni et al. [6]

4. Primers are used which create in-frame fusion with the Fc gene in pBIOCAM5-GW. This is achieved by introducing *Nco*I and *Not*I sites in the primers, "in frame" with the *Nco*I and *Not*I sites present in pBIOCAM5-GW (*see* Fig. 4a *and also* **Note 1**). Primers are synthesized by Sigma or any oligonucleotide supplier. Store as a 100 μM stock at −20 °C.
5. T4 DNA ligase and reaction buffer containing Tris–HCl and DTT are from Roche. Store at −20 °C.
6. GeneJET PCR and plasmid miniprep purification kits (Thermo Scientific).
7. *Nco*I and *Not*I restriction enzymes (New England Biolabs). Store at −20 °C.
8. Electromax DH5α *E. coli* electrocompetent cells (Lifetech). Store at −80 °C.
9. Gene Pulser electroporation system (Biorad).
10. LB, Agar, SOC medium (Difco).
11. Kanamycin (50 mg/ml stock) (Sigma). Store at −20 °C.

2.3 Materials for LR Recombination of pBIOCAM5-GW Library Entry Plasmid with pGATOR-DTA

1. pBIOCAM5-GW entry plasmid library is prepared as described in Section 3.1. Store at −20 °C.
2. pGATOR-DTA is available from Addgene (plasmid # 64103).
3. Gateway LR Clonase II Enzyme mix and Proteinase K (2 μg/μl) (Lifetech). Store at −20 °C.
4. 1 M Tris–HCl, pH 8 and Ethanol (Molecular Biology grade) from Sigma. Store at room temperature.
5. DH10B *E. coli* chemically competent cells (Lifetech). Store at −80 °C.
6. Circlegrow medium (Anachem). Store at room temperature.
7. *Sfi*I restriction enzyme (New England Biolabs). Store at −20 °C.
8. Ampicillin (100 mg/ml stock, Sigma). Store at −20 °C.
9. Midiprep plasmid DNA kit (Machery Nagel).
10. Phenol:chloroform (Sigma). Store at 4 °C
11. 250-ml baffled Erlenmeyer flask.

2.4 ES Cell Lines

The choice for the appropriate reporter genes depends on which signaling pathway is interrogated at which differentiation stage and at what developmental context. It is preferable to use—when possible—"knock-in" ES cell lines in which expression of the fluorescent, lineage-specific reporter genes of interest is driven by their endogenous promoters. In this protocol mouse ES cell lines are used, but human pluripotent stem cells or induced pluripotent stem cells (iPSCs) can also be used.

Mouse ES cell reporter cell lines appropriate for monitoring the differentiated versus non-differentiated state of a culture are the following:

1. Oct4-ΔPE-GFP ES cells are derived from Oct4-ΔPE-GFP mice, in which GFP is specifically expressed in the pre-implantation embryo and primordial germ cells but not the post-implantation epiblast [11]. In these cells GFP is expressed under the control of the distal enhancer of Oct4 and specifically reports for the naïve ES cell state but not the primed, epiblast stem cell (EpiSC) state. This cell cell line was constructed in Azim Surani's lab, the Gurdon Institute, Cambridge, UK (*see* **Note 2**).
2. Nanog-GFP ES cells (TNGA), in which GFP is inserted in the *Nanog* locus, one of the key "pluripotency" genes, and also reports for the naïve ES cell state [12]. This cell line was constructed in Austin Smith's lab, Welcome Trust Stem cell Institute, Cambridge, UK.
3. Rex1-GFPd2 ES cells, in which GFP is inserted in the *Nanog* locus [13]. Rex1 gene is regarded as an extremely sensitive marker of pluripotency. This cell line was constructed in Austin

Smith's lab, Welcome Trust Stem cell Institute, Cambridge, UK (*see* **Note 3**).

2.5 Materials for Embryonic Stem Cell Culture and Differentiation

All work should be carried out in a mycoplasma-free dedicated stem cell culture facility, using standard tissue culture (TC) equipment and plasticware.

1. Gelatin (0.1 % swine skin): Make from master stock (1 %). Store both at 4 °C.
2. Glasgow Minimum Essential Medium (GMEM) (500-ml aliquots, Invitrogen): Store at 4 °C. Discard a 50-ml aliquot to make room for other ingredients of the ES cell medium.
3. Fetal calf serum (FCS): Selected batch to support ES cell self-renewal (Sigma). Store 500-ml bottles at −80 °C. Aliquot one bottle in 50-ml vials and store at −20 °C.
4. Glutamine (200 mM): Prepare from a 100× stock (Invitrogen). Aliquots of 5 ml glutamine are mixed with 2.5 ml Penicillin/Streptomycin and kept at −20 °C.
5. Penicillin/Streptomycin: 200× stock containing 10^4 U/ml penicillin and 10 mg/ml streptomycin/ml (Sigma). Aliquots of 2.5 ml Penicillin/Streptomycin are mixed with 5 ml glutamine and stored at −20 °C.
6. Sodium Pyruvate (Sigma): Aliquot and store at −20 °C.
7. MEM non-essential amino acids: 100× stock (Invitrogen). Store at 4 °C.
8. 2-mercaptoethanol (Sigma): Store at room temperature.
9. LIF-ESGRO at 10^7 U/ml (Invitrogen): Store in 5-ml aliquots at −80 °C and 1-ml aliquots at −20 °C.
10. CHIR99021, a GSK-3 inhibitor and PD0325901, an ERK kinase inhibitor, are from Stratech.
11. Phosphate-Buffered Saline (PBS DULBECCO'S) free of Mg^{2+} and Ca^{2+} (Invitrogen). Store at 4 °C.
12. 0.05 % Trypsin/EDTA (Invitrogen): Store at 4 °C. Prewarm at 37 °C before use.
13. N227, or NDiff-N227- previously known as NDiff-N2B27- (Stem Cell Sciences). Store 500-ml aliquots at −80 °C. Store 50-ml aliquots at −20 °C.
14. 16 gauge blunt-end needles and 6 cc luer lock syringes.
15. ES-Cult M3120 (Stem Cell Technologies): Make aliquots and store at −20 °C (*see* **Note 4**).
16. Stem Cell Medium: GMEM medium, 15 % FCS, 1 mM Sodium Pyruvate, 2 mM L-Glutamine, 1× MEM non-essential amino acids, 0.1 mM β-mercaptoethanol, 100 U/ml recombinant LIF, 100 U/ml penicillin, 0.1 mg/ml streptomycin,

1 μg/ml doxycycline (when appropriate). Store stem cell medium at 4 °C and use within 3 weeks.

17. Defined (serum free) ES Cell medium (2i-LIF): N227, 1 μM CHIR99021, 1–2 μM PD0325901, 100 U/ml recombinant LIF. Aliquot N227 in 50-ml vials and store at −20 °C. Thaw each vial just before use at 37 °C, or O/N at 4 °C. Add fresh CHIR99021, PD0325901 and LIF just before use. Store remaining medium at 4 °C.
18. ES Cell Differentiation medium (ES-Cult/N227): 60 % v/v N227, 40 % v/v ES-Cult M3120, 1 μg/ml puromycin, when appropriate (*see* Section 3.4), 1 μg/ml doxycycline (where appropriate, *see* Section 3.6). Make aliquots (*see* **Note 4**) and store at −20 °C. Thaw O/N at 4 °C before use.

2.6 Materials for ES Cell Transfection and Positive Selection of Targeted Clones

1. All materials required for ES cell culture as stated in Section 2.5.
2. Mouse ES Cell Nucleofector kit (Lonza). Store at 4 °C.
3. Nucleofector II (Amaxa Biosystems).
4. Puromycin, 10 mg/ml (Sigma). Store at −20 °C.

2.7 Materials for Isolation and Propagation of Individual Undifferentiated ES Cell Colonies from Semi-Solid (Differentiation) Medium

1. All materials required for ES cell culture as stated in Section 2.5.
2. Prepare the following 96-well plates: (a) to one flat-bottomed, pre-gelatinized 96-well plate, add selection medium (120 μl) with a multichannel pipette (MCP) and keep the plate in a 37 °C incubator; (b) to another round-bottomed 96-well plate, add 40 μl of PBS/trypsin solution (3:1, v/v) with an MCP.

2.8 Materials for Isolation of ES Cell Genomic DNA from 96-Well Plates

1. Multichannel pipette (MCP).
2. Trypsin/PBS solution (1:2 v/v).
3. Lysis Buffer: 10 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 % (w/v) SDS and 0.5 mg/ml Proteinase K.
4. Isopropanol.
5. Ethanol 70 %.
6. TE buffer, pH 8.0.
7. Thermo-pierce lids (Merch).
8. SORVAL centrifuge with 96-well plate adaptors.

2.9 Materials for ES Cell Freezing in 96-Well Plates

1. For ES cell growth and trypsinization materials, *see* Section 2.5.
2. Freezing medium: 50 % (v/v) FCS, 20 % (v/v) DMSO, 30 % (v/v) ES cell medium.
3. Parafilm (Sigma).

2.10 Materials for Recovery of Antibody Genes from ES Cell Clones

1. Genomic DNA (*see* Section 3.8). Store at 4 °C.
2. KOD Hot Start Polymerase and buffer and $MgSO_4$ (25 mM) from EMD Millipore. Store at −20 °C.
3. pBIOCAM5 scFv-Fc expression plasmid, available from Addgene (plasmid #39344). Store at −20 °C.
4. DMSO (Hybri-Max grade, Sigma). Store at RT.
5. Primer 1: 5′-CTTTCTCTCCACAGGCGCCATGG-3′ and primer 2: 5′-GTGTGGGTCTTGTCTGCGGC-3′. Store as a 100 μM stock at −20 °C (*see* **Note 5**).
6. For other PCR reagents and equipment, *see* Section 2.2.
7. *E. coli* BL21(DE3) chemically competent cells (New England Biolabs). Store at −80 °C.
8. For restriction enzymes and agarose electrophoresis reagents, *see* Section 2.2.
9. GeneJET PCR purification kit and GeneJET gel extraction kit (Thermo Scientific).

2.11 Materials for Expression and Purification of Selected scFVs

1. Reagents and materials for cell inoculation (*see* Section 2.3).
2. HEK293 freestyle cells and medium (Lifetech).
3. JetPEI (Polyplus).
4. PBS (pH 7.4) (Invitrogen). Store at RT.
5. Imidazole, 1 M stock (Sigma).
6. Ni-NTA sepharose (Qiagen).
7. Proteus "1 step batch" midi spin columns (Generon).
8. GeBaFlex dialysis tubes (Generon).

3 Methods

3.1 Cloning of Antibody Genes into pBIOCAM5-GW Entry Vector

As a first cloning step, the preselected antibody library (in the form of a single-chain variable fragment (scFvs)) is cloned into pBIOCAM5-GW entry vector, in order to create a fusion with a human IgG1 Fc gene (scFv-Fc fusion) (*see* Fig. 4a). The Fc domain causes dimerization of the resultant antibodies. pBIOCAM5-GW vector also has the advantage that it harbors Gateway recombination sites, which facilitates the subsequent step involving incorporation into the targeting vector (*see* Section 3.2).

1. A glycerol stock of *E. coli* TG1 cells harboring the antibody phage-display plasmid, containing preselected antibody populations to the target of interest, is scraped with an inoculating loop and transferred to a microfuge tube containing sterile water (100 μl). The optical density at 600 nm (OD_{600}) is measured with a spectrophotometer and a volume of cells

a Sequence of pBIOCAM5-GW

```
              NcoI                 XhoI                    NotI
GGC GCC ATG GCC CAGATCCTCGAGGACATCCAC GCG GCC GCA GAC AAG ACC
CCG CGG TAC CGG GTCTAGGAGCTCCTGTAGGTG CGC CGG CGT CTG TTC TGG
 G   A   M   A                         A   A   A   D   K   T
___"Leader_____                                   --Fc domain->
```

b Typical incoming scFv

```
      NcoI                                                       NotI
CCG GCC ATG GCC GAG GTG CAG CTG TTG /// CGA CTG GAG ATT AAA GCG GCC GCA
GGC CGG TAC CGG CTC CAC GTC GAC AAC /// GCT GAC CTC TAA TTT CGC CGG CGT
 P   A   M   A   E   V   Q   L   L -  R   L   E   I   K   A   A   A
__Leader________I I______VH___________>   <_______VL_________I
```

Fig. 5 (**a**) Sequence of pBIOCAM5-GW showing position and framing of *Nco*I and *Not*I cloning sites. (**b**) Typical incoming scFv gene showing *Nco*I cloning site, first five amino acids of a VH, last five amino acids of VL region and *Not*I cloning site

equivalent to 0.0125 OD_{600} (*see* **Note 6**) is taken as a template for a PCR reaction described in step 2.

2. Antibody genes are amplified by PCR using as template 0.01 OD_{600} *E. coli* prepared according to step 1. The PCR mix also contains primers (5 μM each), 1× HotStarTaq DNA polymerase buffer, 200 μM dNTPs and 2.5 U HotStarTaq DNA polymerase in a total volume of 100 μl. Cycling conditions are 95 °C, 15 min, followed by 30 cycles of 94 °C, 0.5 min; 54 °C, 0.5 min and 72 °C, 1 min. The product is analyzed by 1 % agarose, TBE gel electrophoresis. If a single band of 750–800 bp is produced, this can be purified using a PCR purification kit. PCR primers introduce *Nco*I and *Not*I sites for cloning. It is important that these are in frame with the *Nco*I and *Not*I sites present in pBIOCAM-GW (*see also* Fig. 5).
3. scFv PCR product (2.5 μg) is digested with *Nco*I and *Not*I (50 U each) for 6 h at 37 °C and purified by PCR purification kit.
4. pBIOCAM5-GW (10 μg) is digested with *Nco*I and *Not*I (50 U each) for 3 h at 37 °C and purified by 1 % agarose, TBE gel electrophoresis.
5. Ligation reactions are performed between the pBIOCAM5-GW vector produced in step 4 and the scFv inserts produced in step 3. Ligation reactions consist of 400 ng pBIOCAM5-GW vector, 100 ng scFv insert, 66 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM DTT, 1 mM ATP and 1 U T4 DNA ligase in a total volume of 20 μl. The ligation mixture is incubated at 16 °C for 16 h, purified by PCR spin column and eluted with 30 μl HPLC grade water.

6. Purified ligation mix from step 5 (1.5 μl) is used to transform 20 μl electrocompetent *E. coli* DH5α by electroporation in a 0.1-cm electroporation cuvette, using the Gene Pulser electroporation system (EC1 setting). After addition of 1 ml of SOC medium and incubation at 37 °C, with shaking, 20 μl is plated onto a 10-cm diameter LB agar plate, containing 50 μg/ml kanamycin (LB-Kan) to enable the counting of individual colonies to estimate library size and the remaining transformation mix plated onto five separate LB-Kan plates and incubated at 37 °C for 18 h. The total library size should be between 10^5 and 10^7.
7. The LB-Kan antibody library plates are harvested by the addition of 2 ml 2TY medium to each plate and scraped with an L-shaped spreader and the *E. coli* culture transferred to a 50-ml falcon tube. The OD_{600} is measured and a volume of cells equivalent to 10 OD_{600} units is harvested for miniprep plasmid DNA preparation. This yields purified pBIOCAM5-GW plasmid DNA harboring antibody library genes.

3.2 LR Recombination of pBIOCAM5-GW Library Entry Plasmid with pGATOR-DTA to Produce the Final Targeting Construct

In this second cloning step, Gateway recombination is used to facilitate the introduction of the scFv-Fc library into a 10-kb region of the mouse *Rosa*-26 gene of the pGATOR-DTA targeting vector (Fig. 4b) to create the final targeting construct (Fig. 4c). In this construct, the use of a "Tet-On" transcriptional activation system affords the opportunity for temporal control of antibody expression [6].

1. A recombination reaction is performed by incubating 29 ng pBIOCAM5-GW library, entry plasmid prepared according to Section 3.1 and 312 ng pGATOR-DTA in 1× LR clonase II diluted in TE (pH: 8) buffer in a total volume of 10 μl. The reaction is incubated for 18 h at 25 °C.
2. Add 1 μl of 2 μg/μl Proteinase K solution and incubate at 37 °C for 10 min. The reaction mix is purified by PCR clean-up spin column, eluted with water (30 μl). Add 10 μl of purified recombination reaction to 90 μl chemically competent *E. coli* DH10B cells on ice and incubate for 30 min. Heat shock at 42 °C for 45 s and add 1 ml SOC, rescue at 37 °C for 1 h, and plate 20 μl onto a 10-cm LB-Amp plate to estimate library size and the remainder onto five LB-Amp plates and incubate at 37 °C overnight.
3. The next day scrape the LB-Amp agar plates as described in Section 3.1, step 7. Add the retrieved *E. coli* to 60 ml of circlegrow medium containing 100 μg/ml ampicillin in a 250-ml baffled Erlenmeyer flask and incubate at 210 rpm (25 mm orbital throw), 37 °C for 5 h until $OD_{600} = 8$.
4. Harvest the cells and prepare plasmid (pROSA-ic) by midiprep.

5. Digest 50 μg pROSA-ic with 150 U *Sfi*I in a total volume of 100 μl for 2 h at 50 °C. The digest is purified by phenol, chloroform extraction and ethanol precipitation. The DNA pellet is washed with 70 % ethanol and dissolved in 25 μl TE (pH: 8). The concentration of linearized pROSA-ic should be 1–2 μg/μl.
6. Run a *Sfi*I-digested pROSA-ic aliquot on an agarose gel to confirm full digest, as linearization of the targeting construct is essential for homologous recombination to proceed efficiently.

3.3 Mouse ES Cell Culture

All work should be carried out in a mycoplasma-free dedicated stem cell culture facility, using standard tissue culture (TC) equipment and plasticware.

1. Gelatinize TC plates by applying 0.1 % gelatine enough to cover the surface area. Leave at room temperature for at least 10 min. Aspirate the gelatine prior to plating of the cells.
2. A vial containing ES cells in ES cell medium plus 10 % DMSO is removed from liquid nitrogen and put immediately at 37 °C. When the vial is half-thawed, rapidly transfer the content to a universal tube containing 5 ml of ES cell medium and centrifuge at 272 × *g* for 5 min in a benchtop centrifuge. DMSO-containing medium is removed as soon as possible and the cells are resuspended in ES cell medium and plated on gelatine-coated plates at ~1×10^6 cells/6-well.
3. ES cells are fed every day by aspirating half of the old medium and replacing it with new. During growth the ES cells are observed under a phase, inverted microscope to monitor their growth rate and check for signs of differentiation.
4. The cells are ready to passage when they reach near confluency, usually every 2–3 days, usually between 1:5 and 1:8 ratio. To passage the cells grown in the presence of serum, wash the plate twice with PBS (2 ml per 6-well) and add trypsin/EDTA (0.5 ml solution to a 6-well). A 3-min incubation at 37 °C follows, after which the plate is tapped until the cells come off. ES cell medium, containing FCS to inactivate the trypsin, is added to a final volume of 1.5 ml per 6-cm plate (3 volumes of medium) and the cells are pipetted up and down with a 1-ml Pasteur pipette, until the cell clumps become dissociated. The cells are then inspected under an inverted-phase contrast microscope to ensure that a single cell suspension has been achieved. ES cell medium is added to a final volume of 5 ml and mixed with the cells. The cells from one plate were then re-seeded onto five different fresh plates (1:5 split ratio). To passage ES cells grown in serum-free medium the process is the same, except that 10 volumes of ES defined medium are

added to dilute the trypsin solution after 3 min of incubation and the trypsinized cells are centrifuged at 272 × *g* for 5 min. Remove the supernatant and resuspend the ES cells in ES medium, as stated above.

5. To freeze the ES cells, trypsinize as described above and resuspend in ES cell medium containing 10 % DMSO (v/v). Transfer the cells in freezing vials (~2.5 × 10^6 per vial). Transfer the vials to −80 °C and 1–24 h later to liquid nitrogen, for long-term storage.

3.4 Targeting Antibody Genes into the ROSA-26 Locus of Mouse Embryonic Stem Cells and Selection of Targeted Clones

The linearized targeting construct (*see* Section 3.2) is introduced into mouse ES cells, whereby homologous recombination between the homologous *Rosa*-26 sequences of the construct and the endogenous locus in the ES cells leads to integration of the antibody genes in the ubiquitously expressed *Rosa*-26 chromosomal locus (1 gene integration/per ES cell on average).

1. Transfect mouse ES cells with the linearized final targeting construct by nucleofection using the Mouse ES Cell Nucleofector kit and program A-023 of Nucleofector II (Amaxa Biosystems).
2. Use linearized DNA (3.8 μg) to transfect 4.5 × 10^6 ES cells and plate the transfected cells into two 100-mm dishes containing ES cells medium.
3. After 24 h and for the next 4–5 days, administer 1 μg/ml puromycin to commence positive selection. Change the medium every day to remove dead cells.
4. Following selection single puro^r ES colonies are either picked and propagated under self-renewal conditions for further analysis/storage or subjected to differentiation conditions (*see* Section 3.6).

3.5 Practical Considerations Dictating the Design of the ES-ICER Differentiation Assay

1. Identifying functional antibodies with the ES-ICER method requires that target antigens have a clearly defined effect on the differentiation state of the cells. Based on this knowledge a robust and unambiguous differentiation assay is then designed to probe for the perturbation of that effect by the functional antibody (*see* Fig. 2).
2. The selection of the differentiation assay also determines the selection of the appropriate reporter ES cell line (*see also* Section 2.4). In most cases it is convenient if a positive outcome is reported for, e.g., proliferation, or survival/maintenance of the reported cell population (*as in* Fig. 2b, *see also* **Note 7**).
3. Targeting an antibody population to the ES cells results in the integration of a single antibody gene into the *Rosa*-26 locus per ES cell. It is, therefore, important to ensure the physical

separation of transfected ES clones to avoid diffusion of the secreted antibodies, which would dilute the effect of the potential efficient blockers derived from a few elite ES colonies. Depending on the time frame of the differentiation protocol, various strategies can be employed to achieve this: For relatively short differentiation assays (of 3–5 days) antibody-secreting ES colonies may be maintained in a methylcellulose-containing, semi-solid medium, in which individual antibody populations are prevented from freely diffusing away from the encoding ES cell colony. Functional antibody-expressing colonies exhibiting the desired phenotype may be manually picked and individually propagated. Alternatively, clonal isolation of functional antibody-expressing cells may be achieved by serial process of fluorescence-activated cell sorting and re-culturing of the isolated population under differentiation conditions. After 3–4 days of culture in semi-solid medium, ES cell colonies start to detach from the plate, and therefore, when longer differentiation screening assays are required, a change in strategy is needed. In these cases, transfection, positive selection and initial differentiation of a pool of clones may take place, followed by fluorescent-activated sorting of relevant progenitor cells. These progenitors may be further subject to directed differentiation, either in semi-solid medium or in 96 wells, where inducible expression of functional antibodies from individual colonies will affect their differentiation and allow for phenotypic selection.

3.6 In Vitro Differentiation Assay to Identify Puro^r ES Cell Clones Expressing Unique scFv-Fcs, Which Inhibit Their Differentiation Under Differentiation Conditions in Semi-Solid Medium

The basic protocol can be adapted, to be used in conjunction with different reporter cell lines and different target antigens. As an example a differentiation assay used for the identification of antibodies inhibiting FGF4/FGFR signaling is described here. When self-renewal signals are deprived (under differentiation conditions), autocrine FGF4 binds to FGFR1β receptor mediating the initial differentiation signal to ES cells through the activation of MEK-ERK signaling pathway [14–17]. Based on this knowledge the experiment is designed to identify ES colonies expressing inhibitory anti-FGFR1β antibodies by their ability to remain undifferentiated when grown under differentiation conditions. In this case a semi-solid medium is used in the initial screening assay, as the distinction of differentiated versus undifferentiated state of the ES cells can be made in a short window frame (3–4 days). To report for the undifferentiated, pluripotent state of the ES cells, the Oct4-ΔPE-GFP ES line is chosen (*see also* Section 2.4).

1. Transfect Oct4-ΔPE-GFP ES cells with an anti-FGFR1 preselected antibody phage-display library and apply positive selection as described in Section 3.4.

2. After 4 days of puromycin selection and 36 h prior to differentiation initiation, add doxycycline (*see* **Note 8**), to initiate antibody gene expression under TRE control within the ROSA-26 gene prior to the autocrine production of FGF4 from ES cell colonies. Include negative control (cells grown in the absence of doxycycline) and positive control (cells grown under self-renewal conditions, in 2i/LIF).
3. At the fifth day of selection, replace ES medium with the serum-free (*see* **Note 9**), semi-solid differentiation medium, containing doxycycline (1 μg/ml) where appropriate. As mentioned above, the role of MCM is to assign to the medium a considerable viscosity, in order to prevent the different populations of antibodies from diffusing and mixing in the medium.
4. Monitor the differentiation progress daily by observing the morphology and the GFP fluorescence of the colonies (*see* **Note 10**). The maximum time the colonies can be sustained in semi-solid medium without detaching from the dish is around 4 days. This timing is sufficient to allow for discrimination between colonies retaining and those downregulating Oct4-GFP fluorescence. After 3–4 days a few ES cell colonies retaining Oct4-ΔPE-GFP expression together with a compact, round shape, typical of an undifferentiated ES cell colony, should be readily distinguishable from the bulk of differentiating colonies under the fluorescent microscope (*see* Fig. 6).
5. Flow cytometry analysis can also be performed at this stage to confirm the observations of the morphological inspection and assess the potency of the antibody population in a more quantitative way.
6. Proceed with either manual isolation of the desired colonies (*see* Section 3.7). Alternatively, potent antibodies can be isolated through serial fluorescent-activated sorting of the GFP-positive cell pool followed by rounds of differentiation in semi-solid medium.

3.7 Isolation and Propagation of Individual Undifferentiated ES Cell Colonies from Semi-Solid Medium

To isolate and propagate the desired colonies expressing potent antibodies, carry out the following steps:

1. Mark the location of desired colonies in the dish with a pen under a fluorescent inverted microscope.
2. Pick the selected colonies from one differentiation plate under a phase, inverted microscope (preferably inside the hood) with a Gilson pipette, set at 20 μl, into the wells of the round-bottomed 96-well plate containing the PBS/trypsin solution. (Use a fresh box of tips during this procedure to keep track of the number of colonies picked).

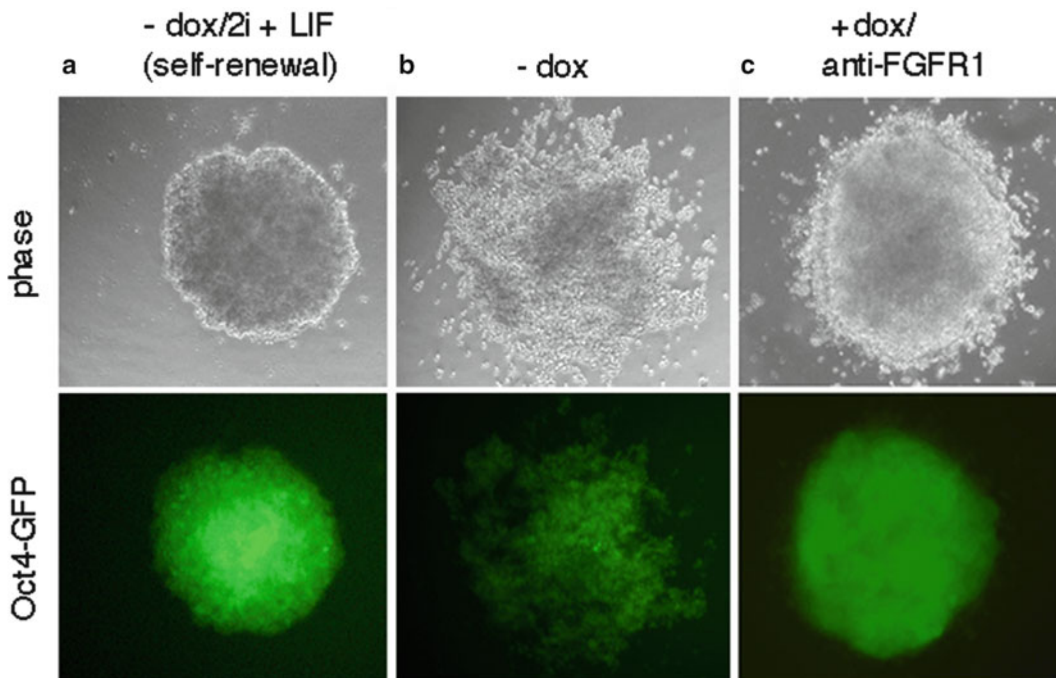

Fig. 6 Morphology and fluorescence profiles of Oct4-GFP-ΔPE ES cells transfected with an anti-FGFR1-β population after 3 days of differentiation. (**a**) Typical undifferentiated ES cell colony grown in serum-free ES cell medium in the absence of doxycycline (positive control). (**b**) Typical differentiated colony in the absence of doxycycline (negative control). Note the loss of compactness and round shape, indicative of loss of E-cadherin-mediated cell adhesion. Also the marked decrease in GFP fluorescence intensity and extent, indicative of the downregulation of expression of the Oct4 pluripotency marker. (**c**) Example of an elite colony preserving an ES cell-like compact shape and rounded morphology and maintaining high levels of GFP fluorescence at a large degree all around the colony. This colony expresses an anti-FGFR1-β antibody that blocks the FGF4-mediated signaling normally leading to differentiation. Figures taken from Melidoni et al., (2013) *Proc Natl Acad Sci U S A* 110(44): 17802–7, with permission from PNAS

3. Incubate the multi-well plate at 37 °C for 3 min; transfer to the hood and mix colonies vigorously with a multichannel pipette, then transfer to the flat-bottomed 96-well plate containing ES cell medium containing 0.5 μg/ml puromycin. Incubate the plate in the 37 °C incubator.
4. Follow standard ES culture to grow the ES cell colonies. Only ES colonies that had remained truly undifferentiated under the previous differentiation conditions will be able to propagate. Therefore, this step provides a second check point in the selection process of ES colonies expressing antibodies that inhibit their differentiation.
5. After a few days when the wells are confluent or semi-confluent, split the wells to make duplicate 96 wells, one for DNA isolation (*see* Section 3.8) and one (master plate) for freezing at −80 °C (*see* Section 3.9). Selected clones are further

propagated to undergo a secondary differentiation assay in the presence of doxycycline, followed by flow cytometry analysis to quantitate the effect of the expression of the antibodies in the expression of the reporter markers, which will reflect the potency of the antibodies.

3.8 Isolation of ES Cell Genomic DNA from 96-Well Plates

1. Use a multichannel pipette in all steps.
2. Remove the spent medium and wash the cells once with 100 μl of PBS.
3. Add 40 μl of trypsin/PBS solution (1:2 v/v) and incubate the plates at 37 °C for 3 min.
4. Pipette the cells thoroughly to disperse and transfer into the wells of another 96-well plate containing 100 μl of lysis buffer.
5. Seal the plates with nylon film and incubate overnight at 37 °C.
6. The next day add isopropanol (100 μl) directly to the lysates, to precipitate the genomic DNA in the presence of endogenous salts.
7. Seal the wells with thermo-pierce lids and turn them upside down gently several times, until DNA strands are visible.
8. Centrifuge for 30 min at 2000 × *g* in a SORVAL RT7 centrifuge, after which the DNA forms a visible pellet.
9. Place the plates inverted on tissue paper until most of the liquid is removed.
10. Add 50 μl of 70 % ethanol, followed by a 15-min centrifugation at 2000 × *g*.
11. Invert the plates again, as before, to get rid of excess liquid and re-centrifuge inverted for 2 min at 95 × *g*. This additional step removed all the liquid, while the DNA was allowed to stack in the bottom of the wells.
12. The plates are then left at room temperature to dry completely and the DNA is resuspended in 20 μl of TE, pH 8.0. The plates are kept at 4 °C.

3.9 ES Cell Freezing in 96-Well Plates

1. When cells are confluent, remove the spent medium and wash the cells twice with 50 μl of PBS.
2. Add trypsin solution (40 μl) and incubate the plates at 37 °C for 3 min.
3. Tap the plates to facilitate dislodging of the cells
4. Add freezing medium (100 μl) to the mixture and pipette a few times to disperse the cells.
5. Seal the plates with parafilm, place in a polystyrene box and transfer to −70 °C.

3.10 Recovery of Antibody Genes from ES Cell Clones

1. Set up a PCR reaction in KOD buffer with 100 ng genomic DNA, 100 μM dNTPs, 1.5 mM $MgSO_4$, 5 % (vol/vol) DMSO, 0.4 U KOD Hot Start Polymerase, and primers 1 and 2 (0.25 μM each) in a total volume of 20 μl. Cycling conditions are 95 °C, 2 min followed by 40 cycles of 95 °C, 20 s; 57 °C, 10 s; 70 °C, 40 s.
2. Purify PCR products with PCR purification kit.
3. Digest PCR products with *Nco*I and *Not*I and purify by electrophoresis on a 1 % TBE-agarose gel.
4. Excise the band running at approximately 800 bp and extract the DNA using GeneJET gel extraction kit.
5. Digest the scFv DNA insert with *Nco*I and *Not*I and ligate with the *Nco*I/*Not*I-digested single chain antibody expression plasmid pBIOCAM5 vector [18].
6. Transform chemically competent *E. coli* BL21(DE3) and plate onto LB agar plates supplemented with ampicillin (100 μg/ml).

3.11 Expression and Purification of Selected scFv-Fc Antibodies

1. Inoculate 1 ml of LB supplemented with 100 μg/ml ampicillin with a single amp^r colony generated in Section 3.10 and grow 6 h at 37 °C in a shake incubator to generate a starter culture.
2. Inoculate 60 ml of circlegrow medium, containing 100 μg/ml ampicillin, with 600 μl of starter culture from step 1 in a 250-ml baffled Erlenmeyer flask and incubate at 210 rpm (25 mm orbital throw), 37 °C O/N.
3. Prepare plasmid DNA using any standard midiprep plasmid DNA kit.
4. Transfect HEK293 suspension cells with 50 μg DNA and 100 μl of PEIpro per 50 ml HEK293F cells at 10^6 cells/ml.
5. Harvest culture supernatant 5 days post transfection by transfer to a 50-ml falcon tube and centrifugation at 2000 × *g* for 10 min.
6. Pipette 45 ml of clarified culture supernatant into a 50-ml falcon tube containing 5 ml 10× PBS (pH 7.4), 10 mM imidazole. Add 0.3 ml of Ni-NTA sepharose (50 % slurry) and mix by end over end rotating for 1 h.
7. Centrifuge the culture supernatant/bead mix at 1000 × *g* for 10 min. Remove the culture medium by pipetting and resuspend the Ni-NTA sepharose with 5 ml of wash buffer (2× PBS (pH 7.4), 20 mM imidazole) and transfer to Proteus "1 step batch" midi spin column prewashed with PBS. Centrifuge at 200 × *g* for 2 min and discard the wash.
8. Add 5 ml wash buffer, spin at 200 × *g* for 2 min and discard the flow through.

9. Repeat step 8 once.
10. Add 0.3 ml elution buffer (2× PBS, pH 7.4, 300 mM imidazole), incubate for 2 min, and spin at 200 × *g* for 2 min. Collect the eluate.
11. Repeat step 10 three times or until the antibody has fully eluted as measured by absorbance at 260 nm.
12. Pool the eluate fractions and dialyze against 2 × 4 L PBS, pH 7.4 with midi GeBaFlex dialysis tubes.
13. Measure antibody concentration by absorbance at 260 nm and store at 4 °C or −80 °C for long-term storage.

4 Notes

1. The following primers were used when using antibody populations selected using the *McCafferty* antibody phage-display library: M13 LeadSeq (5′-AAATTATTATTCGCAATTCCTTTGGTTGTTCCT-3′) and NotMycSeq (5′-GGCCCCATTCAGATCCTCTTCTGAGATGAG-3′). When amplifying single chain antibodies from alternative libraries, care should be taken to ensure that *Nco*I and *Not*I sites are added to be compatible with reading frame encoded in pBIOCAM5-GW.
2. Oct4-ΔPE GFP ES cell line is the preferred cell line, when using a fluorescent microscope to monitor levels of Oct4-ΔPE GFP expression, since fluorescence is much more intense than Nanog-GFP and particularly Rex-GFP ES cell lines.
3. The fluorescence of this cell line can only be monitored by flow cytometry, since the unstable form of GFP used in the construct (GFPd2) makes it unsuitable for fluorescent microscopy.
4. Handle medium with a blunt-end needle attached to a syringe ONLY (and not with a pipette), as it is very viscous, due to the methylcellulose present in ES-Cult M3120.
5. Primers for antibody gene recovery are designed to amplify the single chain (scFv) antibody genes directly are located at the immediate beginning and end of the scFv coding sequence, adjacent to the *Nco*I and *Not*I cloning sites.
6. An $OD_{600} = 0.0125$ of *E. coli* culture is equivalent to 10^7 cells, which is roughly a tenfold oversampling of the selected antibody population, assuming 10^6 individual clones are present after two rounds of antibody phage-display selection.
7. There is the potential to report loss of the reporter cell type but this relies on having low background for this event.

8. This allows sufficient time for the anti-FGFR1 molecules to be produced and secreted around each ES colony. Potent antibodies are expected to shield ES cells from the upcoming autocrine-produced FGF4 that would be induced as a result of the removal of the self-renewal signals.
9. The differentiation medium is serum free to avoid the presence of any excess FGF4, or other factors that could potentially activate the MEK/ERK signaling pathway, apart from the ES-derived autocrine FGF4. Remove any traces of serum present in the ES cell medium by washing the plates twice with GMEM before applying the semi-solid differentiation medium to the ES colonies (day 0 of differentiation).
10. Keep a separate test dish with differentiating cells of samples and positive and negative controls to observe under the microscope every day and the original dishes left undisturbed in the CO_2 incubator for the whole differentiation period. Due to the methylcellulose-containing medium, moving of the plates to and from the microscope causes the colonies to become detached from the dishes and start floating in the medium).

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Delivering Antisense Morpholino Oligonucleotides to Target Telomerase Splice Variants in Human Embryonic Stem Cells

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Abstract

Morpholino oligonucleotides (MO) are an innovative tool that provides a means for examining and modifying gene expression outcomes by antisense interaction with targeted RNA transcripts. The site-specific nature of their binding facilitates focused modulation to alter splice variant expression patterns. Here we describe the steric-blocking of human telomerase reverse transcriptase (hTERT) Δα and Δβ splice variants using MO to examine cellular outcomes related to pluripotency and differentiation in human embryonic stem cells.

Keywords: Morpholinos, Human embryonic stem cells, Telomerase, Telomerase splicing, Differentiation

1 Introduction

Gene knockdown is an experimental technique that is commonly used to elicit changes in the expression pattern of one or more candidate targets. Knockdown can be accomplished using a variety of techniques, including: small double-stranded interfering RNAs (siRNA), short-hairpin RNAs (shRNA), Peptide nucleic acid (PNA), Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs), and morpholino oligonucleotides (MO). MO are a class of oligomers that bind small regions of RNA in an antisense orientation and can be used to modify gene function through expression inhibition, altering RNA splicing, and by changing miRNA activity and maturation (1–4). In contrast to methods like RNAi and shRNA, MO do not require the intracellular machinery for action, and with the ability to steric block specific splice junction sites, offers an effective tool to alter cellular gene expression and function.

With this technology, our group has successfully inserted steric-blocking MO into human embryonic stem cells (hESCs) (3, 4). This technique was used to reveal the potential biological roles for specific TERT splice variants in pluripotency and self-renewal. Specifically, antisense MO complementary to specific exon–intron or intron–exon boundaries of the pre-mRNA sequence of TERT were

used to block alternative splicing events in this transcript. Utilizing a fluorescently tagged MO during mechanical cell passaging, we demonstrated efficient uptake of MO into human embryonic stem cells. From this application we were able to demonstrate, for the first time, the specificity and effectiveness of MO for targeting TERT pre-mRNA to alter the TERT splice variant expression pattern in pluripotent stem cells.

2 Materials

All solutions and media should be prepared and handled in sterile conditions. For solutions that cannot be autoclaved, filter sterilization is recommended to eliminate contamination. For specific details related to culture components, *see* Table 1.

2.1 hESC Culture Components

1. Human embryonic stem cells (hESC): fully validated and quality controlled cell lines can be obtained from a range of companies. In our previous study (4), and in the examples discussed here, we utilized the H9 hESC line that can be purchased from WiCell (WiCell Research Institute; Madison, WI).
2. hESC Culture Medium: fully validated and quality controlled hESC media can be obtained from numerous sources. In these works we utilize a defined, growth factor supplemented medium called mTeSR1 for Culture of hESCs on Matrigel™. H9 hESCs were grown in hESC medium in all experiments where MEF feeder cells were utilized. hESC medium composed of Knockout

Table 1
List of the materials/reagents utilized, suppliers, and catalogue numbers

Material/reagent	Supplier	Cat. No.
mTeSR1	StemCell Technologies	05850
Knockout Dulbecco's modified Eagle's medium (DMEM/F12)	Invitrogen	12660-012
Knockout serum replacement (KOSR)	Invitrogen	10829-018
Nonessential amino acids	Invitrogen	11140-050
L-glutamine	CellGro	25-015-CL
Matrigel™ (Growth Factor Reduced)	BD Biosciences	354248
2-mercaptoethanol	Fisher Scientific	BP176100
Basic fibroblast growth factor (bFGF)	Invitrogen	PHG0261
hESC-qualified GIBCO® Geltrex	Invitrogen	A10480-02
Ultra-Low Cluster Plates	Corning	3471

Dulbecco's modified Eagle's medium (DMEM/F12), 20 % Knockout serum replacement (KOSR), 1 % nonessential amino acids, 2 mM glutamine, 0.1 mM 2-mercaptoethanol, and 4 ng/mL of basic fibroblast growth factor (bFGF).

3. hESC Culture Surface: hESCs require a substrate for growth beyond conventional tissue-culture treated dishes. The basement membrane matrix Matrigel™ is commonly used as a supplemental growth surface for hESCs. In these works we utilized a growth factor reduced version of Matrigel™. Alternatively, hESC-qualified GIBCO® Geltrex can be used in place of Matrigel™. Although culture of hESCs can be carried out on irradiated layers of embryonic mouse fibroblasts, we recommend the use of more defined matrices to limit confounding of experimental results from feeder cells.
4. hESC Culture Dishes: We utilized Ultra-Low Cluster Plates from Corning in a six-well format for all experiments discussed here.

2.2 Morpholino Design and Preparation

1. MO sequences should be designed such that they are complementary to the target pre-mRNA molecule of interest. To improve the strength and specificity of binding, oligonucleotides that span a distance of 25 nucleotides are recommended (MO works by targeting splice junctions or splice regulatory sites). In this study MO (Gene Tools, LLC; Philomath, OR) complementary to specific exon–intron or intron–exon boundaries of the pre-mRNA sequence of TERT were used to specifically block the splicing of the Δα splice (intron 5/exon 6) and Δβ splice intron/intron 8) variants of TERT (Fig. 1). As controls, antisense MO that contains no specificity to any transcript should be utilized. All MO used should carry a fluorescein tag on their 3′ end for visualization and quantification of cellular uptake efficiency.
2. Resuspend lyophilized MO from the manufacturer (Life Tools, LLC; Philomath, OR) in sterile water at a concentration of

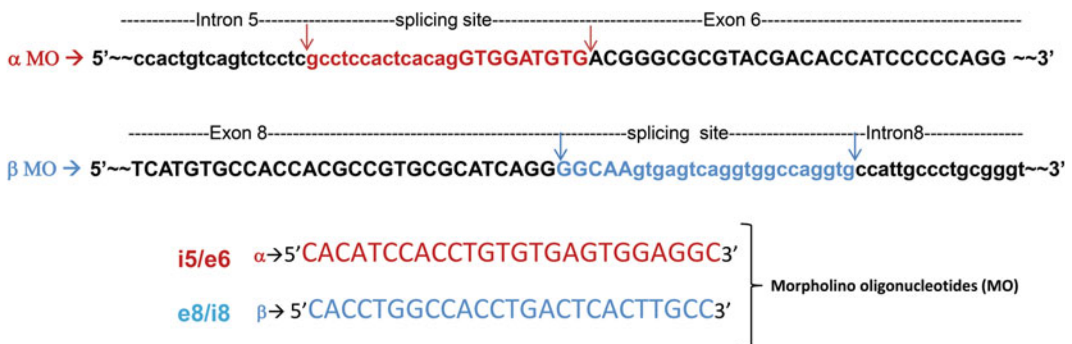

Fig. 1 Morpholino oligonucleotides designed for steric-blocking the Δα and Δβ TERT splice variants. The MO sequences are designed from 5′ to 3′ to be complementary to specific intron–exon or exon–intron boundaries of the pre-mRNA sequence of TERT that blocks the alternate splice sites Δα (intron 5/exon 6—*red*) and Δβ (exon 8/intron 8—*blue*) (*see* **Note 11**)

1 mM (as recommended by the manufacturer). Vortex the solution briefly. This stock solution may be stored at room temperature temporarily, or at −20 °C indefinitely. MO solutions should be warmed to room temperature for at least 2 h prior to use. Depending on the target, a dose curve should be carried out to determine the optimal working concentration for efficient steric-blocking of each specific splicing event targeted.

3 Methods

Carry out all procedures at room temperature and inside fume hood at all times. Medium should be pre-warmed to 37 °C for at least 1–2 h prior to use unless otherwise specified.

3.1 Preparation of Matrigel™ Coated Plates

1. Slowly thaw the Matrigel™ stock solution on ice at 4 °C for at least 2 h prior to starting. It is important to thaw Matrigel™ slowly, as rapid warming will cause gelation of the matrix.
2. Prepare the Matrigel™ coating solution by mixing Matrigel™ and basal DMEM/F12 medium at a 1:30 ratio in 15 mL falcon tube. Mix this solution gently using a 1 mL pipette (*see* **Note 1**).
3. Coat the whole surface of each new culture plate by pouring or pipetting 1 mL of the Matrigel™ coating solution into each well of a six-well dish. Rotate the plate gently to obtain even coverage of the well surface (*see* **Note 2**).
4. Incubate the coated wells in an incubator on a level surface at 37 °C and 5 % CO_2 incubator for 1–2 h to promote gelation.

3.2 Culture of hESCs on Matrigel™ Coated Plates

1. hESCs from a frozen stock can be plated on Matrigel™ coated plates as prepared above, or a feeder layer of mouse embryonic fibroblasts (MEF). We recommend plating hESCs from a frozen stock onto feeder cells prior to passaging onto Matrigel™ plates. hESCs cultured on MEFs can be grown in standard embryonic stem cell medium supplemented with basic fibroblast growth factor (*see* Section 2).
2. From a confluent dish of hESCs grown on MEFs, aspirate the medium from the six-well tissue culture plate (*see* **Note 3**).
3. Rinse each well to be passaged once with 1 mL of pre-warmed mTeSR1 medium (*see* **Note 4**).
4. Add 2 mL of pre-warmed mTeSR1 medium in each well to be passaged.
5. Mechanically pick colonies into small sizes with a *glass-picking device* using a stereoscope placed in a sterile hood (*see* **Note 5**).
6. Gently release the hESCs from the plate with gentle pipetting using a 5 or 10 mL glass pipette. Transfer the contents of each well into a 15 mL falcon tube (*see* **Note 6**).

7. Re-dissociate any hESC clumps that may have aggregated into individual clusters by gentle pipetting with a 1 mL tip. Take care to not reduce the solution to a single cell suspension.
8. Plate a predetermined amount of hESCs depending on the desired passage ratio of mTeSR1 containing the picked cells into each well of the Matrigel™ coated six-well plate (*see* **Note 7**).
9. Add 1 mL of additional fresh, pre-warmed mTeSR1 medium to each well.
10. Place the dish in a humidified incubator at 37 °C and 5 % CO_2 in air atmosphere. Ensure the cells are evenly distributed by moving the plate back-and-forth on a level surface.
11. Leave the plate undisturbed for 24 h to facilitate attachment. Change the culture medium every 24 h for the duration of the culture.

3.3 Transfecting hESCs with MO

1. Prepare a culture dish by coating each required well with Matrigel™ as described above. Keep this plate in an incubator at 37 °C prior to use.
2. From the dish containing the cultured hESCs ready for MO treatment, aspirate the medium from each well (*see* **Note 8**). Rinse each well to be treated once with 1 mL of pre-warmed mTeSR1 medium.
3. From the aspirated wells, add 500 μl of pre-warmed mTeSR1 and incubate for 10–15 min at 37 °C to equilibrate the cells prior to MO treatment (*see* **Note 9**).
4. During the previous steps incubation, gently aspirate the medium from the Matrigel™ coated plate that was prepared previously (*see* Section 3.3, **step 1**). Add 1.5 mL of pre-warmed mTeSR1 medium per well, and place the dish back in the incubator.
5. Add an appropriate concentration of the MO stock solution (*see* Section 2.2) to each well to be treated in the dish containing the hESC colonies.
6. Mechanically pick hESC colonies in small sizes under the microscope in the presence of the MO (*see* Section 3.2 and **Note 6**).
7. Stir wells by rotating the dish very gently and wait for about 30 s to increase exposure of MO to hESC.
8. Gently dissociate the picked hESCs from the well with a 5 or 10 mL glass pipette and gently transfer the contents of each well into one in the coated Matrigel™ plate prepared previously (*see* Section 3.3, **step 4** and **Note 10**).
9. Place the plate into a 37 °C, 5 % CO_2 incubator and move it in short, back-and-forth and side-to-side motions to disperse cells across the surface of the dish.
10. Leave the plate for 24 h to facilitate hESC attachment.

11. Feed hESCs daily by aspirating spent medium and replacing with fresh mTeSR1 containing the utilized concentration of each MO. Repeat this procedure for a total of 3 days following the initial treatment.

In order to determine the efficiency of transfection of the MO, visualize the hESCs under a fluorescent microscope 24 h after first MO treatment. Depending on the tag utilized, fluorescence should be evenly visible throughout the cells (*see* Figs. 2a and 3a). Efficiency can also be quantified using flow cytometry (*see* Fig. 2b).

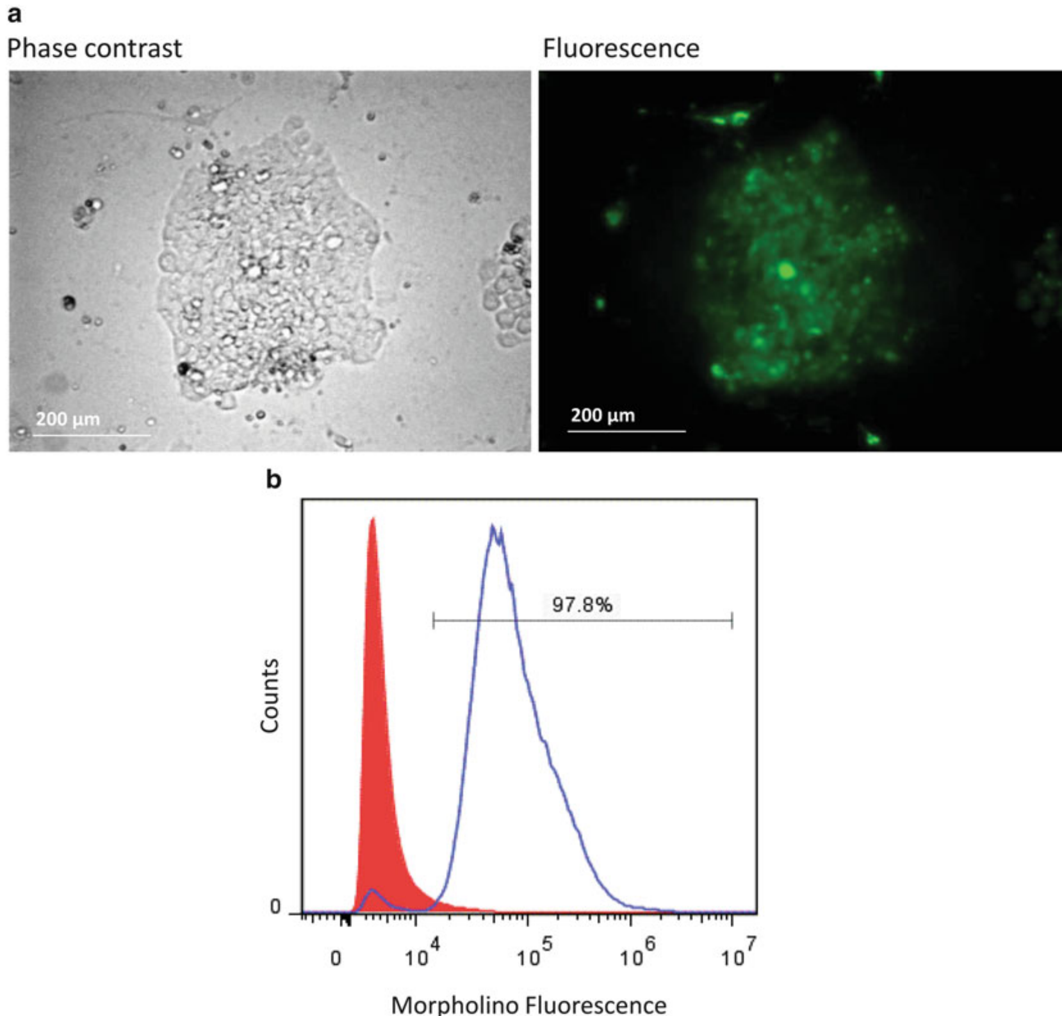

Fig. 2 MO fluorescence in cultured hESCs and the efficient uptake of MO into hESCs as measured by flow cytometry. (**a**) Fluorescein-tagged morpholino molecules are observed by live cell fluorescence microscopy and appear as green fluorescence in hESCs. (**b**) A representative flow cytometry analysis of hESCs containing fluorescein-tagged scrambled control MO. We have achieved up to 97.8 % efficiency of MO uptake indicating efficient MO delivery into hESCs (H9 line) with our protocol. Percentage within the bar (**b**) indicates the number of cells positive for MO fluorescence

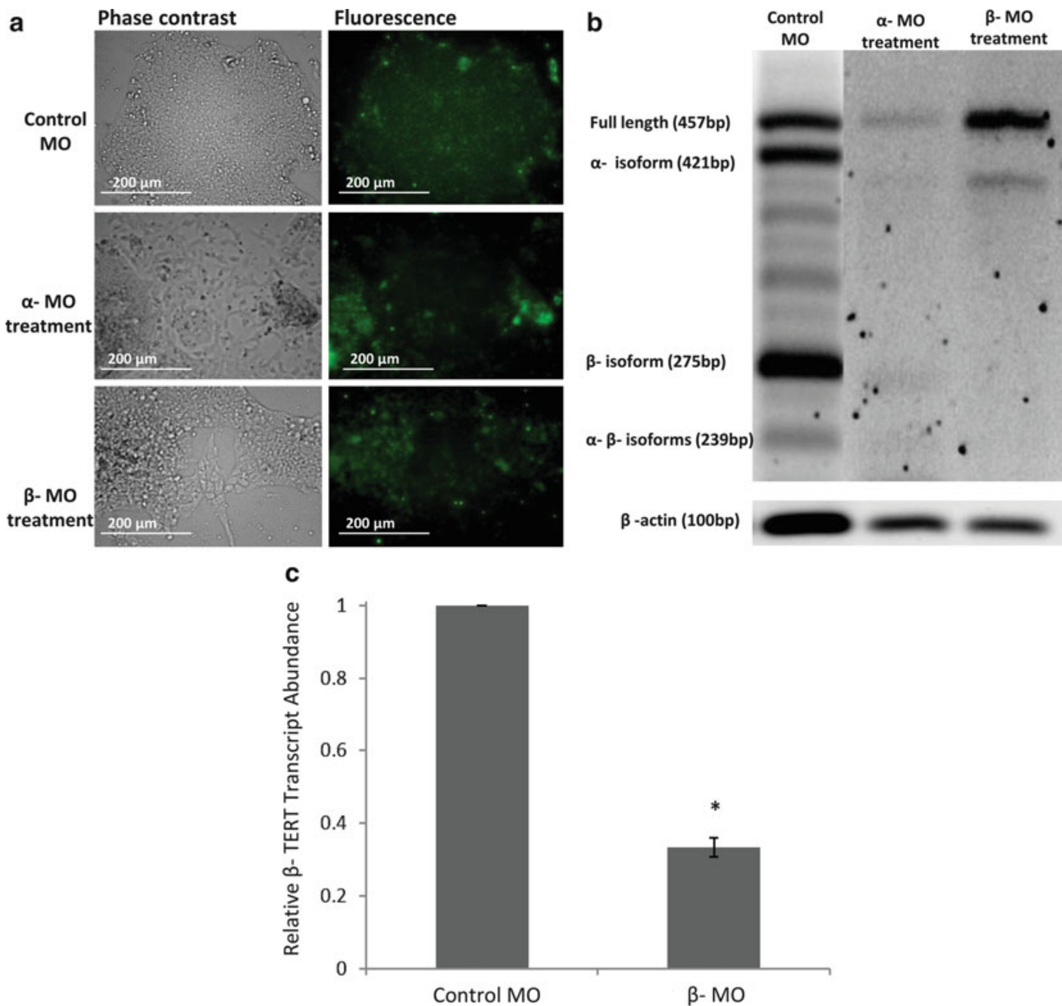

Fig. 3 Steric-blocking MO alters TERT splicing events in hESCs. (**a**) Steric-blocking MO specific for intron 5/ exon 6 and exon 8/intron 8 were effectively taken up into hESCs. Fluorescently labeled MO as observed by live cell fluorescence microscopy. MO appears as green fluorescence within hESCs. (**b**) Example of in-gel verification of MO activity in TERT isoform expressing hESCs. The displayed agarose gel electrophoresis of PCR amplified cDNA samples demonstrates a decrease in transcript abundance of specific TERT isoforms following treatment with either intron 5/exon 6 or exon 8/intron 8 MO's compared to standard control MO-treated hESCs (Adapted figure panels **a** and **b** reprinted with permission from STEM CELLS & DEVELOPMENT, 2014, by Radan et al., published by Mary Ann Liebert, Inc., New Rochelle, NY). (**c**) Example of quantification of specific splice variant (Δβ hTERT) expression by hydrolysis probe RT-qPCR indicating altered expression about the splice site bounded by exon 8/intron 8

3.4 Characterization of Effective Steric-Blocking and Modification of Splicing Events by MO

Proper characterization of the splice variant transcript pattern produced following morpholino exposure is crucial to the interpretation of results. The effectiveness of the targeted activity of the MO can be assayed using either standard RT-PCR followed by agarose gel electrophoresis (Fig. 3b), or hydrolysis probe RT-qPCR (Fig. 3c). Typically splice donor or acceptor sites at

intron–exon boundaries are blocked with MO's causing the spliceosome to use a nearby alternative donor or acceptor site. Ideally MO blocking leads to an increase in the desired alternative splicing; however, this may also lead to several possible unexpected effects that should be kept in mind when analyzing results (5–7). Off-target effects include the use of alternative donor/acceptor sites or the use of cryptic splice sites leading in some cases to full or partial intron inclusion or deletion of exons that are not endogenously targeted. Thus, careful analysis of the splice-patterns post MO treatment must be carried out.

We carried out standard RT-PCR (4), which has been the "gold standard" in evaluating the abundance of alternative splice transcripts (Fig. 3b). RT-PCR has the advantage of detecting intron inclusion and other normally undesired effects. However, RT-PCR relies on the end-point measurement of fluorescence and without extensive optimization suffers from nonlinearity in the typical range of visibility. Conversely, hydrolysis probe-based RT-qPCR can be conducted to accurately quantitate the transcript abundance of specific hTERT splice variants (Fig. 3c), but may not detect, or may misrepresent, undesired splice events without careful analysis. Careful design of primers/hydrolysis probes can allow quantification of all combinations of two or three adjacent splicing sites providing the maximum product size does not exceed qPCR capabilities. It is beneficial to carry out both RT-PCR and hydrolysis probe RT-qPCR as complementary methods providing an initial confirmation of specificity of splicing, and an accurate quantitation of its effectiveness. We have used a hydrolysis probe splicing scheme as previously described (8) that is able to measure all combinations of two splice sites ($\Delta\alpha/\Delta\beta$) by using four groupings of two forward and two reverse primers, and one FAM-BHQ1a tagged hydrolysis probe (Eurofins Operon). Alongside careful splice variant transcript characterization following targeted MO treatment, pluripotency marker detection, proliferation/apoptosis rates, and other analyses of pluripotent stem cell function should be evaluated depending on the chosen splice target(s) that are modified (4).

3.5 Summary and Future Perspectives

Morpholino's offer a highly efficient method of altering pre-mRNA splicing and gene expression patterns in hESCs, having been previously validated useful in embryo and cancer research (9, 10). MO's are a useful addition to the existing genomic toolbox allowing easy targeting of genes both at the pre-mRNA splicing level as well as the translation level. In combination with other technologies such as RNAi, CRISPR, TALENs, ZFN, and overexpression, MO's can help to elucidate gene function in pluripotent stem cells. As with other genome tools, nonspecific effects such as off-target binding should be closely monitored.

4 Notes

1. Matrigel™ will solidify rapidly once at room temperature. It is important to work quickly with a prepared solution of Matrigel™ and to keep all solutions chilled on ice. If noticeable aggregates have formed in the Matrigel™ solution, it is best to chill the solution until the formations disappear. If the Matrigel™ solution solidifies, it can be recovered by overnight incubation on ice at 4 °C.
2. When using a six-well dish, 1 mL of coating solution is sufficient for each well. If plates of different well sizes are used, adjust the amount of coating solution accordingly to provide sufficient coverage of the well.
3. It is recommended to passage hESC colonies that display sharp edges and consist of uniform colonies of compact cells (Fig. 2). Overgrown colonies and cultures will not passage well. Avoid colonies where differentiation has begun, denoted by the loss of sharp edges and the appearance of flattened cells.
4. When rinsing each well, add and aspirate the medium slowly to avoid detaching healthy hESC colonies. The rinse facilitates the removal of detached colonies.
5. For a picking device, we typically use a glass Pasteur pipette that has been pulled to a pointed tip using a flame. Other devices, such as a sterile syringe needle can also be used. Passaging tools, such as the StemPro® EZPassage™ Disposable Stem Cell Passaging Tool can also be utilized. However, in our experience the yield during the MO reactions was higher when picking mechanically with a glass pipette.
6. For the purpose of MO transfection, it is better to passage hESCs by mechanical picking rather than enzymatic dissociation. In addition, it is advantageous to retain a small colony size during passaging to increase the number of cells exposed to the outer edge of the colony where transfection will be more efficient. When picking, a good strategy is to cut a colony into an even grid with each clump containing approximately 10–30 hESCs. When detaching the picked colonies from the MEF layer, take care to not release cells from the feeder layer that will then be carried to the next dish.
7. In general, a single confluent well of a six-well dish is expected to contain ~1×10^6 hESCs. In the case of confluent wells, a passaging ratio of 1:6 is acceptable, leading to the transfer of ~1.5×10^5 cells per well. However, for preparing colonies for MO transfection, it is better to use a 1:2 ratio in order to obtain small, confluent wells after 2 days of culture.

8. Ensure colonies are healthy and undifferentiated prior to starting the MO protocol. Healthy colonies should have sharp, defined borders and no flattened differentiated cells around the edges (Fig. 2a).
9. This is the medium that will be used during the MO treatment. It is best to use a defined, serum-free media to minimize potential unspecific binding of the MO to the components of the medium.
10. It is ideal to passage the hESCs such that they will remain at a high confluence in the secondary culture, while trying to avoid over confluence. As a starting point, use a passaging ratio of 1:1. In this culture, check for viability and efficiency of MO transfection. If successful, lower passaging ratios can be utilized to eliminate any potential deleterious effects arising from over confluence.
11. The intron 5/exon 6 MO will block the intron 5 splice acceptor region and cause use of the intron 6 splice acceptor (results in exon 6 deletion), or the alpha-splice variant acceptor site (results in 36 bp deletion in exon 6 = $\Delta\alpha$). Inclusion of part of intron 5 is an undesired possibility. The exon 8/Intron 8 MO blocks the splice donor of intron 8. Possible outcomes include use of the intron 7 donor (results in exon 8 deletion), or use of the intron 6 donor (causing exon 7 and 8 deletion = $\Delta\beta$ splicing to be used). Undesirable outcomes can include partial intron 8 inclusion.

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Analysis of mRNA Translation Rate in Mouse Embryonic Stem Cells

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Abstract

Regulation of gene expression is essential to enable embryonic stem cells (ESCs) to either self-renew or to differentiate. Translational regulation of mRNA plays a major role in regulating gene expression and has been shown to be important for ESC differentiation. Sucrose gradients can be used to separate mRNAs based on the number of associated ribosomes and this can be used as a readout of the rate of translation. Following centrifugation through a sucrose gradient, mRNAs can be recovered, purified, and analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) to determine their ribosomal load in different cell states. Here, we describe how to differentiate mouse ESCs to Neural Precursor Cells (NPCs) and analyze the rate of translation of individual mRNAs by qRT-PCR following polysome fractionation.

Keywords: Stem cells, Translation, Polysome, Differentiation, Quantitative real-time PCR

1 Introduction

Embryonic stem cells (ESCs) have the unique ability to self-renew or to differentiate into multiple different cell lineages. This is driven by changes in gene expression that are coordinated at many levels. Regulation of gene expression is essential for directed differentiation and it includes regulation of transcription, RNA stability, translation, and protein stability. In ESCs, global profiling of the translation state of mRNAs has demonstrated widespread regulation at the level of translation highlighting the limitation of analyzing mRNA levels as a readout of gene expression (1–3). Translational control of an mRNA is regulated largely by the binding of RNA binding proteins or miRNAs (4–6). These trans-acting factors generally target translation initiation and thus decrease the ribosomal load on an mRNA. The number of ribosomes associated with an mRNA can be used as a readout of its rate of translation. mRNAs heavily loaded with ribosomes are considered to be highly translated while those with fewer ribosomes are considered to be nontranslated or inefficiently translated. While current advances in sequencing technologies allow the analysis of ribosome footprints on a genome wide level (7), analysis of the translation rate of

individual mRNAs can be done using sucrose gradients to separate the mRNAs based on size. When cytoplasmic mRNAs are spun through a linear sucrose gradient, mRNAs heavily loaded with ribosomes sediment into the heavy polysome fractions while the mRNAs with fewer ribosomes fractionate with the lighter polysome fractions. The mRNAs can then be isolated and quantitative real-time PCR (qRT-PCR) can be used to determine the relative ribosomal load of individual mRNA species. The use of splice variant specific primers enables the analysis of differences in translation rate between different splice variants within the same cell or between different cell states.

Mouse ESCs can be efficiently differentiated to Neural Precursor Cells (NPCs) using established protocols (8, 9). These NPCs can then be further differentiated into the three neural sub-types neurons, astrocytes and oligodendrocytes. The protocol detailed here describes how to differentiate mouse ESCs into NPCs using published protocols (8, 9) and then to analyze the ribosomal load of individual mRNA species as a readout of translation rate in the two cell types. This method can be used on a wide range of cell types including both adherent and suspension cell cultures.

2 Materials

All glassware and plasticware should be RNAse free. Autoclave glassware at 120 °C for 30 min prior to use. Prepare all solutions using nuclease, protease, and pyrogen free biotechnology grade water and analytical grade reagents. To protect against RNA degradation, decontaminate workbench with RNase Away followed by DEPC water before the start of experiment. Wear gloves and use barrier tips for all steps of this protocol. Ensure proper waste disposal in accordance with waste disposal regulations.

2.1 Cell Culture Reagents

1. DMEM (Gibco® BRL, Bethesda, MD, USA).
2. ES Fetal Bovine Serum (ES FBS) (Gibco® BRL, Bethesda, MD, USA).
3. β-mercaptoethanol (Gibco® BRL, Bethesda, MD, USA).
4. L-glutamine (100×) (Gibco® BRL, Bethesda, MD, USA).
5. MEM nonessential amino acids (100×) (Gibco® BRL, Bethesda, MD, USA).
6. ESGRO® Leukemia inhibitory factor (LIF), 10^7 U/mL (Merck Millipore ESG1107).
7. Neurobasal® Medium (Gibco® BRL, Bethesda, MD, USA).
8. DMEM/F-12 Medium (Gibco® BRL, Bethesda, MD, USA).
9. N-2 Supplement (100×) (Gibco® BRL, Bethesda, MD, USA).

10. B-27® Supplement (50×), serum free (Gibco® BRL, Bethesda, MD, USA).
11. 0.1 % Gelatin in water (STEMCELL Technologies Inc., Vancouver, British Columbia, Canada).
12. 0.25 % Trypsin–EDTA (Gibco® BRL, Bethesda, MD, USA).
13. Corning® 100 × 10 mm petri dish with cover.

2.2 Preparation of Cell Culture Medium

1. DMEM for ESCs: Prepare 500 mL of ESC culture medium by supplementing ES DMEM with 15 % FBS, 0.2 mM β-mercaptoethanol, 2 mM L-glutamine, 1× MEM nonessential amino acids, and 1,000 U/mL of LIF.
2. N2B27 medium for Neural Precursor (NP) cells: Prepare 400 mL of NP cell culture medium by supplementing with 730 μL of β-mercaptoethanol, 2 mL of N-2 supplement, 1 mL of L-glutamine, 4 mL of B-27 supplement in a (1:1) mix of Neurobasal®–DMEM/F-12 medium.

2.3 Polysome Extraction Components

1. Diethylpyrocarbonate (DEPC) (Sigma Chemical Company, St. Louis, MO, USA).
2. DEPC (0.1 %) treated deionized water.
3. Dulbecco's Phosphate-buffered saline (DPBS), without magnesium and calcium (Gibco® BRL, Bethesda, MD, USA).
4. 10 % Triton X-100: 1 mL Triton X-100 top up to 10 mL with water.
5. 10 % Tween 20: 1 mL Tween 20 top up to 10 mL with water.
6. 10 % sodium deoxycholate stock: Dissolve 10 g of sodium deoxycholate in 100 mL water using a magnetic stirrer (*see* **Note 1**) (Sigma Chemical Company, St. Louis, MO, USA).
7. 1 M Tris–HCl, pH 7.4: Add 2 mL of Tris–HCl (pH 8) (Ambion®, Austin, Texas) to 8 mL of Tris–HCl (pH 7) (Ambion®, Austin, Texas).
8. Sucrose (Sigma Chemical Company, St. Louis, MO, USA).
9. Cycloheximide (100 mg/mL), Ready-made solution (Sigma Chemical Company, St. Louis, MO, USA).
10. 5 M NaCl (Ambion®, Austin, Texas).
11. 1 M $MgCl_2$ (Ambion®, Austin, Texas).
12. 2 M KCl (Ambion®, Austin, Texas).
13. Heparin (10 mg/ml) stock: Add 0.01g of heparin sodium salt to 1mL RNase free water (Sigma Chemical Company, St. Louis, MO, USA).
14. 20U/μL SuperaseIn (Ambion®, Austin, Texas).
15. 10 % SDS solution (Gibco® BRL, Bethesda, MD, USA).

16. RNAse free Proteinase K (20 mg/mL) (Gibco® BRL, Bethesda, MD, USA).
17. TRIzol® Reagent (Ambion®, Austin, Texas).
18. 50 mL syringes.
19. 10 mL syringes.
20. 0.2 μm syringe filters.
21. Long blunt-end needle.
22. RNase Away (Molecular BioProducts Inc., San Diego, CA).

2.4 Preparation of 10 % and 50 % W/V Sucrose

1. 10 % sucrose solution: Weigh 5 g of sucrose in a 50 mL Falcon tube. Add RNase free water to 35 mL. Add 1.875 mL of 2 M KCl, 75 μL of 1 M $MgCl_2$, and 500 μL of 1 M Tris–HCl, pH7.4 to the sucrose solution. Place tubes in boiling distilled water for 15 min, gently agitating the sucrose mix until the sucrose is completely dissolved. Cool on ice. Make volume up to 50 mL with RNase free water and filter through a 0.2 μm syringe filter into a fresh 50 mL Falcon tube.
2. 50 % sucrose solution: Weigh 25 g of sucrose in a 50 mL Falcon tube and prepare a 50 mL sucrose solution as described above. Sucrose solutions can be stored at −20 °C and thawed to room temperature before use.

2.5 Preparation of Buffers and Reagents

1. 2× Resuspension buffer (RSB): Add 200 μL of 1 M Tris–HCl, pH 7.4, 40 μL of 5 M NaCl, 30 μL of 1 M $MgCl_2$ and top up to 10 mL with water. Store at 4°C (*see* **Note 2**).
2. 1× RSB: Add 150 μL of 2× RSB, 16 μL of SuperaseIn (320 U), 0.6 μL of cycloheximide (200 μg/mL), 3 μL of 10 mg/mL heparin and top up to 300 μL with water (*see* **Note 3**).
3. Lysis buffer (LB): Add 500 μL of 2× RSB, 100 μL of 10 % Triton-X, 200 μL of 10 % Tween-20, 100 μL of 10% sodium deoxycholate, 20 μL of heparin (10 mg/mL) and top up to 1 mL with water (*see* **Note 4**).
4. Add 100 μg/mL cycloheximide (1:1,000 dilution) to warm PBS, 0.25 % Trypsin–EDTA, neutralizing medium & ice cold PBS before start of experiment.

2.6 Polysomal RNA Extraction and cDNA Synthesis Components

1. Phenol, pH 6.6–chloroform–isoamyl alcohol (25:24:1) (Ambion®, Austin, Texas).
2. Chloroform–isoamyl alcohol (24:1) (Sigma Chemical Company, St. Louis, MO, USA).
3. 3 M sodium acetate, pH 5.2 (Ambion®, Austin, Texas).
4. Glycogen, 5 mg/mL (Invitrogen®, Carlsbad, CA, USA).
5. 7.5 M Lithium chloride (LiCl) (USB Corporation, Cleveland, OH, USA).
6. Isopropanol (Applichem Biochemica, Germany).

7. 75 % Ethanol in water (Sigma Chemical Company, St. Louis, MO, USA).
8. GeneChip Poly-A RNA Control Kit (Affymetrix, Cleveland, USA).
9. SuperScript® III First Strand Synthesis System for qRT-PCR (Invitrogen®, Carlsbad, CA, USA).

2.7 qRT-PCR Components

1. Power SYBR® Green Master Mix (Applied Biosystems®, Carlsbad, CA, USA).
2. MicroAmp® Optical 384-Well Reaction Plate with Barcode (Applied Biosystems®, Carlsbad, CA, USA).

2.8 Bacterial Spike-in mRNA Primer Sequences

Primers targeting the bacterial RNAs from the polyA RNA control kit.

DAP Forward 5′-CCC ACA GTG ATG ATG TCG AG-3′.

DAP Reverse 5′-GCT GCT TCA GCT GCT TCT TC-3′.

THR Forward 5′-CTC GCT CAA GCT GTC ATG TAC-3′.

THR Reverse 5′-CGG TGA TTT CTC ACA GAT GG-3′.

2.9 Equipment

1. BioComp gradient master model 107ip (BioComp Instruments, Inc. New Brunswick, Canada).
2. Ultracentrifuge model Optima L-Series (Beckman Coulter, Fullerton, CA).
3. SW41Ti rotor and swinging buckets (Beckman Coulter, Fullerton, CA).
4. Seton open-top Polyclear™ Centrifuge Tubes, 14 × 89 mm (BioComp Instruments, Inc. New Brunswick, Canada).
5. Piston gradient fractionator (BioComp Instruments, Inc. New Brunswick, Canada).
6. FC 204 Fraction collector (Gilson, Inc., Germany).
7. Monitor UV-M II (Bio-Rad, Hercules, CA USA).
8. Gradient Profiler Software Version 1.58 (BioComp Instruments, Inc. New Brunswick, Canada).
9. Ultrospec™ 2100 pro UV/Visible Spectrophotometer (GE Healthcare).
10. Thermal cycler (Bio-Rad, Hercules, CA USA).
11. ABI PRISM 7900 Sequence Detection Systems (Applied Biosystems® Carlsbad, CA, USA).
12. Vacuum dryer.
13. Spectrophotometer NanoDrop ND-1000 (Biofrontier Technology).

2.10 Preparation of Centrifuge Buckets and Tubes

1. Chill centrifuge buckets on ice for 30 min before spin. Insert Kimwipes into the bucket to collect condensation. Rinse centrifuge tubes and caps with RNase Away followed by DEPC water and leave it to dry before use. Prechill Eppendorf tubes and Falcon tubes before the start of the experiment (*see* **Note 5**).

3 Methods

All procedures (from Section 3.3) are to be carried out on ice unless otherwise stated. Centrifuges should be prechilled to 4 °C before use.

3.1 Growing ESC and Differentiating Them to NPC

1. Pre-coat 10 cm tissue culture dishes with 0.1 % gelatin 24 h prior to the start of experiment.
2. Seed mouse ESCs at a density of 5×10^6 cells in 2×10 cm dishes in ES DMEM medium 2 days prior to polysome extraction. Incubate cells in a 37 °C, 5 % CO_2 incubator. Change medium 16–20 h before experiment. Harvest cells at no more than 70–80 % confluency (*see* **Note 6**).
3. To differentiate ESCs to NPCs, seed mouse ESCs at a density of 5×10^6 cells in 4×10 cm dishes in N2B27 medium 6 days prior to polysome extraction. Incubate cells in a 37 °C, 5 % CO_2 incubator. Change medium every day.
4. Efficiency of differentiation can be monitored by staining for the neural marker SOX1 in the cells. Efficient differentiations should yield over 80 % SOX1 positive cells.

3.2 Preparation of Sucrose Gradients

Prepare gradients before harvesting cells.

1. Fit 14×89 mm Polyclear Seton tubes into the SW41Ti gradient master tube holder.
2. Add 6 mL of 10 % filtered sucrose to each tube.
3. Use a long blunt needle to gently underlay 5.5 mL of 50 % filtered sucrose at the bottom of the tube being careful to keep the two layers from mixing. Fit in the Biocomp long caps gently to avoid air bubbles and remove any displaced liquid from the cap.
4. Use the Biocomp gradient master as per manufacturer's instructions to make the gradient. Level the machine and choose the program, SW41-List-Long caps-Sucrose-10–50 % w/v.
5. After gradient formation, remove the caps gently (*see* **Note 7**).
6. Weigh the tubes and adjust the weight of the tubes by adding 10 % sucrose to the top of the gradient drop wise.
7. Place the gradients in the chilled centrifuge buckets before use. Gradients must be of equal weight before loading into buckets.

3.3 Polysome Cell Extraction

1. Treat cells with 100 μg/mL cycloheximide for 10 min in 37 °C, 5 % CO_2 incubator (1:1,000 dilution).
2. Wash cells with 5 mL of warm (37 °C) PBS containing 100 μg/mL cycloheximide. Remove PBS and harvest cells with 1 mL of trypsin containing 100 μg/mL cycloheximide. Incubate at 37 °C for 1–2 min. Neutralize trypsin with 4 mL of ice cold medium (with 100 μg/mL cycloheximide). Transfer cells to a prechilled Falcon tube.
3. Spin cells at 380 rcf for 5 min at 4 °C using prechilled centrifuge.
4. Discard supernatant, and resuspend cell pellet in 1 mL of ice-cold PBS (with 100 μg/mL cycloheximide). Transfer cells to a new prechilled 1.5 mL Eppendorf tube.
5. Take out 100 μL of cells and add 1 mL of TRIzol® for total RNA extraction if required.
6. Spin and wash cells twice with 1 mL of ice-cold PBS (with 100 μg/mL cycloheximide) 400 rcf, 4 °C for 2 min.
7. Discard supernatant and resuspend cell pellet in 140 μL of fresh 1× RSB (*see* **Note 8**).
8. Transfer cells (i.e., 160 μL, depends on cell size) to a fresh cold Eppendorf tube. Add an equal volume (i.e., 160 μL) of fresh 1× lysis buffer. Incubate on ice and gently shake the tube every 2 min for 10 min.
9. Spin samples for 3 min at 4 °C, full speed (16,000 rcf) using a microcentrifuge to pellet nuclei (*see* **Note 9**).
10. Transfer supernatant to a new cold tube and spin for 10 min at 4 °C, full speed (16,000 rcf). Transfer supernatant into a fresh tube carefully avoiding the pellet (*see* **Note 10**).
11. Take out 50 μL of extract as unfractionated lysate and add 1 mL of TRIzol® for RNA extraction if required.
12. Take out 10 μL of extract and perform a 1/50 dilution in water to measure the OD units at 254 nm. Prepare 1X RSB at 1/50 dilution in water as blank.
13. Add 400 μL of 10 % sucrose to the top of the gradient gently to avoid mixing of sucrose with the lower layers. Load equal OD units of cell extract onto the sucrose gradients. Top up with 1× RSB if necessary. If sample volume exceeds 250 μL add less sucrose to the top of the gradients.
14. Label the side of each tube with sample name and note which bucket is used.
15. Seal bucket tightly with the lid and spin samples at 8 °C, 36,000 rpm (220,000 rcf) for 1.5 hs in a Beckman ultracentrifuge. Carefully remove the tubes from the rotor and place them in 4 °C fridge.

3.4 Collection of Polysomal Fractions

1. Prepare and label 12 RNase free 1.5 mL Eppendorf tubes per sample on ice. Program the fractionator as follows:

   Speed: 0.3 mm/s, 6.83 mm/fraction, 11 fractions, Total distance: 75.13 mm.
2. Adjust absorbance unit (AU) on software and UV spectrometer monitor according to the sample OD readings (samples with a higher OD reading require a higher absorbance unit) (*see* **Note 11**).
3. Align 1.5 mL Eppendorf tubes on the fraction collector. Collect 11, 1 mL fractions using a gradient piston fractionator simultaneously detecting the absorbance at 254 nm. Manually collect fraction 12 from the bottom of the gradient.
4. Save the UV trace in .csv format (*see* **Note 12**) (Fig. 1).
5. Rinse fractionator between samples using DEPC treated water.
6. After fractionation, add 110 μL of 10 % SDS and 12 μL of 20 mg/mL proteinase K to each fraction.
7. Incubate and shake at 42 °C for 30 min.
8. Samples can be stored at −80 °C or used immediately for RNA extraction.

3.5 RNA Extraction from Polysomal Fractions

1. Bacterial spike-in poly(A) RNAs (containing, DAP and THR mRNAs) are prepared in 1/20 dilution aliquots and stored in −20 °C.
2. 1/20 diluted aliquots are further diluted to 1/100 prior to use. Add 10 μL of diluted spike-in to fresh cold RNase free 2 mL Eppendorf tubes. Transfer 900 μL of polysomal fraction to the tube containing spike-in. Add 1 Volume of 25:24:1 phenol–chloroform–isoamyl alcohol to sample (i.e., 900 μL). Mix well by inverting or vortexing (*see* **Note 13**).
3. Spin at 12,000 rcf at room temperature for 10 min.
4. Transfer 850 μL of aqueous phase into a new RNAse free tube, carefully avoiding the middle layer.
5. Add 1 Volume of 25:24:1 phenol–chloroform–isoamyl alcohol to sample (i.e., 850 μL). Mix well by inverting or vortexing.
6. Spin at 12,000 rcf at room temperature for 10 min.
7. Remove 800 μL of aqueous phase into a new RNAse free tube, carefully avoiding the middle layer.
8. Add 1 volume (800 μL) of chloroform–isoamyl alcohol (24:1). Mix vigorously by vortexing.
9. Spin at 12,000 × *g* for 5 min at room temperature.
10. Remove 700 μL of aqueous phase into a new RNAse free tube on ice.

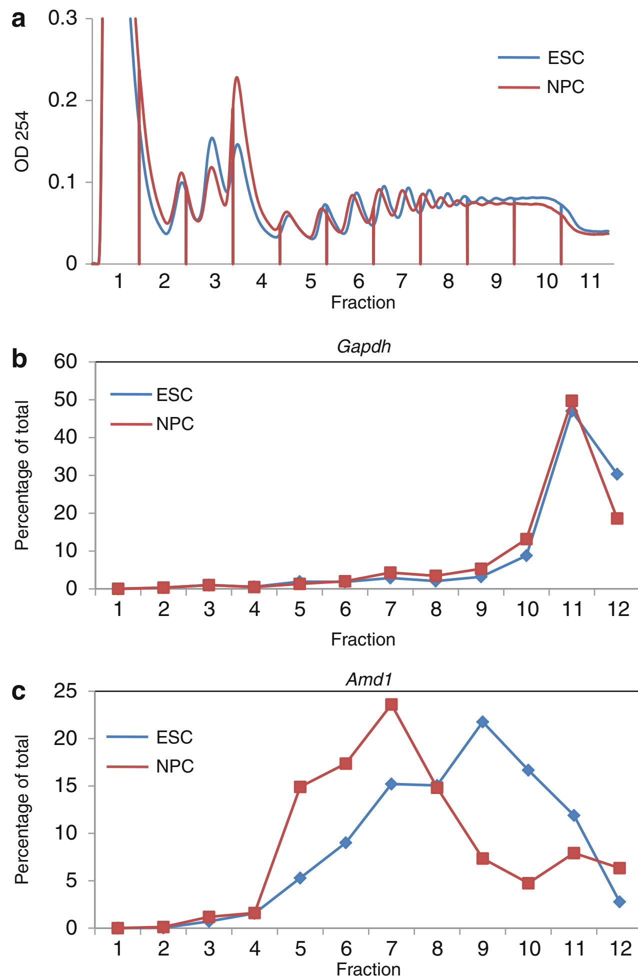

Fig. 1 (**a**) Representative polysome profiles from ESCs and NPCs showing 11 fractions captured on the Biocomp fractionator. Fraction 12 is recovered from the bottom of the gradient. (**b**) qRT-PCR analysis of *Gapdh* and *Amd1* mRNAs in polysomal fractions from ESCs and NPCs. *Gapdh* is similarly loaded with ribosomes in both cell types suggesting an equal rate of translation. *Amd1*, a regulator of the polyamine pathway, is in the heavy fractions (peak in fraction 9) in ESCs and moves to the lighter fractions (peak in fraction 7) on differentiation to NPCs suggesting it is translationally repressed in NPCs (3)

11. Add 1/10th volume of 3 M sodium acetate, pH 5.2 (70 μL) and 3 μL of 2 mg/mL glycogen and mix (*see* **Note 14**).
12. Add 1 volume of cold isopropanol (*see* **Note 15**).
13. Incubate at −80 °C for at least 1 h to overnight (*see* **Note 16**).
14. Spin samples at 4 °C for 30 min at full speed (16,000 rcf) using a microcentrifuge. Look for pellet and carefully discard supernatant.

15. Wash pellet in 700 μL of 75 % ethanol.
16. Spin at 4 °C for 15 min at full speed (16,000 rcf) a microcentrifuge.
17. Resuspend pellet in 50 μL of RNAse free water and add 100 μL of 7.5 M LiCl (*see* **Note 17**). Add 2.5 volumes of 100 % ethanol. Incubate overnight at −20 °C.
18. Spin samples at 4 °C for 30 min at full speed (16,000 rcf) using a microcentrifuge. Carefully discard supernatant. Pellet may not be visible.
19. Wash pellet in 100 μL of 75% ethanol.
20. Spin at 4 °C for 15 min at full speed (16,000 rcf) using a microcentrifuge. Carefully discard supernatant.
21. Resuspend pellet in 100 μL of RNase free water. Add 1/10th volume of 3 M sodium acetate, pH 5.2 (10 μL) and 1 μL of 2 mg/mL glycogen and mix (*see* **Note 18**).
22. Add 2.5 volumes of cold ethanol and mix.
23. Incubate at −20 °C for at least 1 h to overnight.
24. Spin samples at 4 °C for 30 min at full speed (16,000 rcf) using a microcentrifuge. Look for pellet and carefully discard supernatant.
25. Wash pellet in 100 μL of 75% ethanol.
26. Spin at 4 °C for 15 min at full speed (16,000 rcf) using a microcentrifuge. Discard supernatant and dry pellet using a vacuum dryer for 1–5 min (*see* **Note 19**). Resuspend pellet in 30 μL of RNase free water or as desired.
27. Measure RNA concentration and purity using a nanodrop (*see* **Note 20**). RNA can be stored at −80 °C. Avoid repeated freeze thawing of RNA.

3.6 Preparation of cDNA Using Polysomal RNA

1. Calculate the volume for 1 μg of RNA based on the highest concentration among the RNA fractions.
2. Add the same volume of RNA for all the fractions in fresh 0.2 mL PCR tubes. The ng amount will be different for each fraction.
3. Add 1 μL of 1:1 mix of 50 μM oligo$(dT)_{20}$ and 50 ng/μL random hexamers primer and 1 μL of 10 mM dNTP mix, top up to 10 μL with nuclease and RNase free water (*see* **Note 19**).
4. Incubate mixture at 65 °C for 5 min, and cool on ice for at least 1 min.

   Prepare the following cDNA synthesis mix, by adding the reagents in the order as indicated below:

Reagents	1× reaction volume
10× RT buffer	2 μL
25 mM $MgCl_2$	4 μL
0.1 M DTT	2 μL
RNaseOUT™ (40 U/μL)	1 μL
SuperScript® III RT (200 U/μL)	1 μL

5. Add 10 μL of cDNA synthesis mix to the RNA–primer mixture, flick tubes gently and collect by brief centrifugation.
6. Incubate mixture in a thermal cycler as follows:

   25 °C for 10 min, followed by 50 °C for 50 min, and terminate the reaction at 85 °C for 5 min.
7. Store cDNA at −20 °C or use for qRT-PCR immediately.

3.7 qRT-PCR Reaction

1. Dilute 6 μL of cDNA in 194 μL of RNase free water, to make up to 200 μL of diluted cDNA.
2. Prepare primer pair stocks containing 2.5 μM of each gene specific primer in RNase free water.
3. Dilute gene specific primers in Power SYBR® Green master mix at a ratio of 1:5.
4. Load 4 μL of diluted cDNA into each well of a 384-well PCR plate.
5. Load 6 μL of primer/SYBR® Green mix to each well. Centrifuge at 380rcf for 2 min.
6. Load plate into ABI PRISM 7900 Sequence Detection Systems.
7. Perform qRT-PCR reaction as follows:

   Stage 1: 90 °C for 2 min,

   Stage 2: 95 °C for 10 min,

   Stage 3: 95 °C for 30s, 60 °C for 30 s followed by 72 °C for 1 min, for a total of 40 cycles,

   Stage 4: 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s.
8. Normalize raw candidate CT values to spike-in controls DAP and THR. As these were present at equal concentrations in each fraction any differences in the efficiency of RNA extraction between fractions will be reflected in the CT values of these controls. The CT values for the spike-in RNAs should be very similar across all fractions if RNA extractions were equally efficient.
9. Convert resultant CT values to arbitrary units and present relative RNA levels as a percentage of the total RNA where 100 % RNA is calculated as the sum of fractions 1–12 (Fig. 1).

4 Notes

1. Sodium deoxycholate takes a long time to dissolve. It can be stored at room temperature but if precipitates form redissolve before use.
2. 2× RSB can be stored at 4 °C for up to a month.
3. Prepare 1× RSB fresh before the start of the every experiment. The recipe given above is for two sample preps and should be adjusted accordingly.
4. Prepare 1× lysis buffer fresh before the start of every experiment.
5. It is crucial to prechill all Eppendorf, Falcon, and centrifuge tubes as there is increased likelihood of ribosome run-off or RNA degradation if tubes are not kept cold. If polysome peaks do not look as expected on first run, it is likely that the process needs to be done faster and colder.
6. Number of cells seeded should be adjusted as required. Cells should be actively growing, as once they reach stationary phase of growth, translation may be repressed.
7. Avoid tilting the tubes which will disrupt the gradient.
8. Adjust volume of 1× RSB added depending on the size of the cell pellet. A bigger cell pellet may require more than 140 μL of 1× RSB to resuspend.
9. Nuclear pellet will be smaller and whiter than the cell pellet.
10. Take out maximum volume of cell lysate possible. Cell lystate can be snap-frozen and stored at −80 °C for later fractionation.
11. 1.0 AU works best for seven or more OD units of sample loaded. For samples above three OD units, set to 0.5 AU; for samples between one and three OD units, set to 0.2 AU to enhance sensitivity of detection.
12. Open file using an Excel spreadsheet format. Replace the last absorbance value of each fraction with "0" to indicate the end of each fraction. Plot a smooth line of absorbance profile at 254 nm (*y*-axis) against fraction number (*x*-axis).
13. TRIzol® is not recommended for polysome RNA purification from sucrose gradients, and the RNeasy columns can give unequal RNA yields. Phenol–chloroform extraction is recommended with this protocol to enable equal extraction efficiency across all fractions.
14. Glycogen acts as a carrier and helps in the precipitation of RNA.
15. When using isopropanol, the RNA pellet tends to be loose and will be dislodged easily; care should be taken not to disrupt the RNA pellet.

16. Longer precipitation increases RNA yield, and thus, overnight incubation is encouraged. In cases where RNA purity is compromised, an additional ethanol precipitation step is necessary.
17. Heparin inhibits reverse transcriptase activity and has to be removed from RNA samples. Lithium chloride precipitation is performed to remove heparin.
18. Sodium acetate precipitation is performed to remove lithium chloride from RNA.
19. Dry the RNA pellet for about 1–5 min, checking on the pellet every 1 min. Pellet should appear glassy before resuspension. Overdried RNA pellet appears white and will not dissolve easily.
20. For RNA purity, the 260/280 ratio should be over 2 and the 260/230 ratio should be over 1.8.
21. Prepare a master mix of oligoDT, random hexamer primers, and dNTPs according to the number of reactions. Avoid pipetting volumes below 1 μL which may lead to high inaccuracies.

Acknowledgements

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Generation and Purification of Definitive Endoderm Cells Generated from Pluripotent Stem Cells

Ulf Diekmann and Ortwin Naujok

Abstract

Differentiation of pluripotent stem cells into cells of the definitive endoderm requires an in vitro gastrulation event. Differentiated somatic cells derived from this germ layer may then be used for cell replacement therapies of degenerative diseases of the liver, lung, and pancreas. Here we describe an endoderm differentiation protocol, which initiates the differentiation from a defined cell number of dispersed single cells and reliably yields in >70–80 % endoderm-committed cells in a short 5-day treatment regimen.

Keywords: Human pluripotent stem cells, Definitive endoderm, Nodal-signaling, Wnt/beta catenin-signaling, Purification

1 Introduction

Pluripotent stem cells (PSCs) like embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) represent an interesting cell source for cell replacement therapies. PSCs can be differentiated into all adult cell types and consequently may be used in the future for potential treatments of a wide range of degenerative diseases (1). In vitro differentiation of PSCs into somatic cells of the lung (2), liver (3, 4), and pancreas (5–8) requires as a first step the generation of definitive endoderm cells (9, 10). This step is rate limiting as the differentiation efficiency into a certain cell type of the aforementioned organs decreases significantly with each step from pluripotency towards somatic lineage selection. The protocol described here uses a defined number of feeder-free cultured hPSC dispersed into single cells rather than colonies as starting material (9). Dispersed single cells can be differentiated efficiently into the definitive endoderm by a combined treatment with the small-molecule inhibitor of the GSK3, named CHIR-99021, activating the canonical Wnt-signaling pathway, and activin A, activating the Nodal/TGF-β pathway (9). The protocol is independent from PI3K inhibition, which has been used in a number of other studies (8, 11, 12), thereby allowing a robust proliferation and good viability during differentiation (9).

A common consensus exists on how the differentiation efficiency can be analyzed and quantified. This typically comprises the nuclear co-localization of the two core endoderm transcription factors SOX17 and FOXA2 detected by immunofluorescence staining. Characteristic changes in gene expression should also be detectable such as downregulation of pluripotency master regulators, a peaked expression of primitive streak marker genes early during in vitro gastrulation (usually within 24 h), and highly induced gene expression of *FOXA1/2/3*, *SOX17*, *HNF1B*, *GSC*, *CXCR4*, and *GATA* family members upon definitive endoderm commitment (13). In order to quantify endoderm cells, flow cytometry staining of CXCR4 can be used. CXCR4 is a direct target of phospho-SMAD2 and SOX17 (14). Nonetheless, differentiation from pluripotency into endoderm is not 100 % effective and some cells may resist directed differentiation for unknown reasons or differentiate into other unwanted lineages. Additionally, hPSC lines harbor different endoderm formation propensities so that under specific circumstances a purification procedure might be necessary. Thus, this chapter describes two techniques, MACS- and FACS-sorting, to purify/sort endoderm cells to almost 100 % purity.

2 Materials

2.1 PSC Cultivation

1. Matrigel hESC-qualified matrix (*see* **Note 1**) (Corning, Amsterdam, The Netherlands, cat # 354277).
2. mTeSR™1 culture medium (*see* **Note 2**) (STEMCELL Technologies, Vancouver, Canada, cat # 05850).
3. Knockout DMEM/F-12 (Life Technologies, Darmstadt, Germany).
4. Penicillin/streptomycin.
5. Nonenzymatic passaging solution: 0.294 g sodium citrate dihydrate and 23.868 g potassium chloride dissolved in 1 l dest. H_2O. Measure the osmolality; nominal values are 570 ± 10 mOsm/kg. Sterilize by filtration and store at room temperature (*see* **Note 3**) (15).
6. Cell culture-grade plastic plates, dishes, or flasks (e.g., 6-well plates, Corning/Falcon, cat # 353046).
7. Parafilm M.
8. PBS w/o Ca^{2+}/Mg^{2+} (PBS).
9. ROCK inhibitor, Y-27632 (Selleck Chemicals, Munich, Germany, cat # S1049).

2.2 Differentiation

1. Advanced RPMI 1640 (Life Technologies).
2. Glutamax (Life Technologies).

3. Penicillin/streptomycin.
4. CHIR-99021 (Selleck Chemicals, cat # S2924).
5. Human recombinant activin A (Peprotech, Hamburg, Germany, cat # 120-14E).
6. 0.5 % trypsin, 0.2 % EDTA (TE).
7. Primitive streak induction medium: Advanced RPMI 1640, onefold glutamax, onefold penicillin/streptomycin, 0.2 % FBS, 5 μM CHIR-99021, and 50 ng/ml human recombinant activin A.
8. Endoderm induction medium: Advanced RPMI 1640, onefold glutamax, onefold penicillin/streptomycin, 0.2 % FBS, 50 ng/ml human recombinant activin A.

2.3 Flow Cytometry and Cell Sorting

1. Anti-human CXCR4-PE (Neuromics, Minneapolis, MN, USA, cat # FC15004).
2. Anti-human CD49e-FITC (Biolegend, London, UK, cat # 328008).
3. Flow cytometry buffer: PBS w/o Ca^{2+}/Mg^{2+}, 2 % FBS.
4. Sorting buffer: PBS w/o Ca^{2+}/Mg^{2+}, 2 % FBS, 10 μM ROCK inhibitor, 1 mM EDTA.

2.4 Immunofluorescence

1. Anti-human SOX17 raised in goat (R&D systems, Minneapolis, MN, USA, cat # AF1924).
2. Anti-human FOXA2 raised in rabbit (Merckmillipore, Darmstadt, Germany, cat # 07-633).
3. Immunoselect antifading mounting medium with DAPI (Dianova, Hamburg, Germany, cat # SCR-38448).
4. 4-Well/8-well cell culture slides (SPL Life Sciences, Pocheon City, South Korea, cat # 30104/30108).
5. Secondary antibodies (all raised in donkey) conjugated with red or green dyes such as Alexa Fluor 647/Cy5/Daylight 647 or Alexa Fluor 488/FITC/Cy2.
6. 4 % paraformaldehyde (PFA).
7. Donkey serum (Dianova, cat # 017-000-121).
8. Blocking buffer: PBS, 0.2 % Triton X-100, 5 % donkey serum, 1 mg/ml sodium borohydride.
9. Incubation buffer: PBS, 0.1 % Triton X-100, 0.1 % donkey serum.

2.5 qRT-PCR Gene Expression Analysis

1. Trizol, Trifast, or Qiazol reagent.
2. Chloroform.
3. 70 % ethanol.

Table 1
Standard RT-qPCR primer pairs

Gene symbol	Primer sequence 5′-3′	Exon spanning	Accession #
FOXA2	Fw: gggagcggtgaagatgga Rev: tcatgttgctcacggaggagta	Yes	NM_153675.2
G6PD	Fw: aggccgtcaccaagaacattca Rev: cgatgatgcggttccagcctat	Yes	NM_000402
GSC	Fw: gaggagaaagtggaggtctggtt Rev: ctctgatgaggaccgcttctg	Yes	NM_173849.2
MIXL1	Fw: ccgagtccaggatccaggta Rev: ctctgacgccgagacttgg	Yes	NM_031944.1
NANOG	Fw: ccgagggcagacatcatcc Rev: ccatccactgccacatcttct	Yes	NM_024864.2
POU5F1	Fw: cttgctgcagaagtgggtggagg Rev: ctgcagtgtgggtttcgggca	Yes	NM_001173531.2
SOX17	Applied Biosystems Taqman Assay Hs00751752_s1	Yes	NM_022454
SOX2	Fw: agctacagcatgatgcagga Rev: ggtcatggagttgtactgca	Yes	NM_003106.3
T	Fw: tgcttccctgagacccagtt Rev: gatcacttctttcctttgcatcaag	Yes	NM_003181.2
TBP	Fw: caacagcctgccaccttacgctc Rev: aggctgtggggtcagtccagtg	Yes	NM_003194
TUBA1A	Fw: ggcagtgtttgtagacttggaaccc Rev: tgtgataagttgctcagggtggaag	Yes	NM_006009

4. DEPC-treated plastic materials.
5. Column-based RNA purification kit (e.g., Qiagen RNeasy Mini or similar).
6. RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific, Braunschweig, Germany).
7. RNase- and DNase-free water.
8. Random hexamer primer.
9. RNase inhibitor (20 U/μl).
10. 10 mM dNTP mix.
11. PCR primers (10 μM) specific to *SOX17*, *FOXA2*, *T*, *MIXL1*, *GATA4* (*see* Table 1) (9, 10).
12. 2× qPCR gene expression master mix containing SYBR green or similar intercalating DNA dyes.

2.6 Magnetic Cell Separation

1. Anti-human CD184 (CXCR4) MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany, cat # 130-100-070).
2. MACS MS separation columns (Miltenyi Biotec, cat # 130-042-201).
3. 0.5 % trypsin, 0.2 % EDTA (TE).
4. Stopping medium: DMEM, 10 % FBS.
5. Cell strainer 40 μM (BD Biosciences).

6. PB buffer: PBS w/o Ca^{2+}/Mg^{2+}, 0.5 % bovine serum albumin (BSA).
7. PEB buffer: PBS w/o Ca^{2+}/Mg^{2+}, 0.5 % BSA, 2 mM EDTA.

3 Methods

3.1 Feeder-Free Cultivation of Human Pluripotent Stem Cells

3.1.1 Preparation of Matrigel-Coated 6-Well Plates

1. Take a Matrigel aliquot (*see* **Note 1**) from the freezer and thaw it on ice (~1–2 h).
2. Remove the packaging from four 6-well plates and transfer the plates to the bench. Cut four parafilm straps approximately 2 cm wide.
3. Transfer 25 ml ice-cold knockout DMEM/F-12 to a 50 ml conical tube. Transfer the Matrigel solution from the aliquot into the conical tube and rinse the aliquot tube twice with 1 ml ice-cold knockout DMEM/F-12/Matrigel from the conical tube. Ideally the conical tube should be kept on ice.
4. Immediately add 1 ml of DMEM/F-12/Matrigel to each cavity of the 6-well plates. Shake the plates until the surface of each cavity is completely covered with Matrigel. Seal the plates with parafilm to avoid evaporation and store them for up to 7 days in the fridge at 4–8 °C.
5. Prior to the use for passaging the Matrigel-coated cell culture plates must be kept for 30–60 min at room temperature (or alternatively for 15–30 min at 37 °C in an incubator) to ensure polymerization of the matrix (*see* **Note 1**). After polymerization aspirate the supernatant. It can be stored separately for up to 7 days at 4 °C (reused Matrigel). Do not let the well turn dry.

3.1.2 Colony Passaging of Human Pluripotent Stem Cells

1. Aspirate the medium from the cavity of human pluripotent stem cells, which are ready for passaging (ideally 80–90 % confluent) and wash it once with PBS to remove cell debris and dead cells.
2. Add 1 ml nonenzymatic passaging solution to each well and aspirate the solution within approximately 1 min. The colonies should be exposed to a thin liquid film. Transfer the 6-well plate into an incubator (37 °C, 5 % CO_2) and incubate for 3–4 min (*see* **Note 4**).
3. Add 1 ml pre-warmed mTeSR™1 culture medium to each well. Hold the plate and firmly tap the plate 5–10 times to detach the colonies in clusters from the well surface. If the cells still remain on the surface use a blue wide bore cell safer tip and rinse or scrape the colonies from the dish/plate. Optionally you may use a cell scraper. Do not disrupt the clusters into too small fragments or single cells because it will decrease the viability.

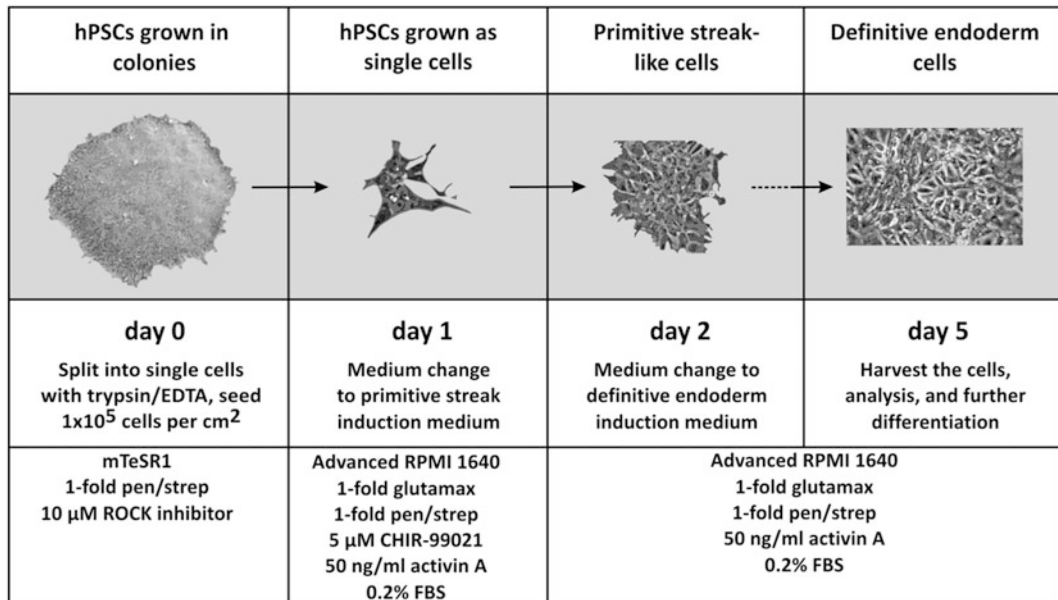

Fig. 1 Schematic illustration of the differentiation of human PSCs into endoderm cells. The three key steps of the definitive endoderm differentiation protocol are depicted. First hPSCs grown in colonies are dissociated into single cells and seeded overnight in culture medium. Subsequently the cells are exposed to high Nodal and Wnt/beta catenin signaling to induce the formation of primitive streak-like cells that harbor endodermal and mesodermal differentiation potential. In vitro gastrulation is further stimulated by activin A/Nodal signaling alone to induce cells reminiscent of the definitive endoderm. Growth factors and medium compositions are given below the respective differentiation stage

4. Transfer the cells carefully to a conical 15 ml tube using a blue wide bore cell safer tip. Add an appropriate amount of mTeSR™1 culture medium to the tube and transfer the cells to the Matrigel-coated 6-well plate. Typically, starting from an optimal density, the cells can be split in a 1:10 to 1:20 ratio. You may optionally adjust the culture medium to 10 μM ROCK inhibitor (Y-27632) to enhance cell/colony survival (*see* **Note 5**).
5. Change the medium every day except the day after passaging. Cultures should be passaged again within 7 days of cultivation.

3.2 Differentiation into Definitive Endoderm

1. A graphical abstract of the differentiation procedure is presented in Fig. 1.
2. Coat a 6-well plate with reused Matrigel surplus from routine PSC cultivation with 1 ml per well (*see* **Note 1**). Keep it for 60 min at room temperature to permit polymerization of the matrix.
3. Aspirate the medium from the wells ready for passaging and wash once with room-temperature PBS.
4. Add 300 μl TE to each well and incubate for 3–5 min in the incubator (*see* **Note 6**).

Table 2
Surface area and cell counts for differentiation

BD multiwell plates and SPL slides, respectively	Surface area	Cell number per well/cavity
6-Well	9.6 cm^2	9.6×10^5
12-Well	3.8 cm^2	3.8×10^5
24-Well	2.0 cm^2	2.0×10^5
4-Well slide	NA	7×10^4
8-Well slide	NA	3.5×10^4

5. Add 1 ml pre-warmed knockout DMEM/F-12 to each well and transfer the cells to a 15 ml conical tube. Optionally, wash the wells with an additional volume of 1 ml pre-warmed knockout DMEM/F-12 and transfer it into the conical tube. Spin down for 300 × *g*/3 min and resuspend in pre-warmed mTeSR™1 culture medium containing 10 μM ROCK inhibitor Y-27632.
6. Count the cells.
7. Seed the cells overnight in a density of approximately 1×10^5 cells/cm^2 (*see* **Note 7**) in mTeSR™1 culture medium with 10 μM ROCK inhibitor Y-27632 onto the coated 6-well plate (*see* point 2. above) or other plate layouts (Table 2).
8. On the following day (approx. 24 h after seeding) change the medium to primitive streak induction medium (*see* **Note 8**).
9. The next day (approx. 48 h after seeding) change the medium to endoderm induction medium. Cultivate the cells for 72 h in this medium with daily medium changes. For the final 24 h of differentiation the FBS concentration can be increased to 2 % to improve viability.

3.3 Quantification of Definitive Endoderm Cells by Flow Cytometry

1. The number of DE-committed cells can be quantified via flow cytometry, e.g., by positivity for the surface marker CXCR4 (16–19). Counterstaining can be performed with labeling of CD49e, which is increased during endoderm commitment although CD49e is not unique to the endoderm (20), and can also be detected in mesodermal cells (10).
2. Harvest DE-committed cells by trypsinization. Add 300 μl TE to each well and incubate for 3–5 min in the incubator.
3. Add 1 ml ice-cold flow cytometry buffer to each well and transfer the cells to a 15 ml conical tube. Spin down for 300 × *g*/3 min, aspirate the supernatant, and resuspend in ice-cold flow cytometry buffer.

4. Count the cells.
5. Adjust the volume and cell count to 200,000 cells/200 μl in flow cytometry buffer. Add 10 μl of CXCR4-PE and 2 μl of CD49e-FITC antibody solution to the cells (*see* **Note 9**). Incubate the cells for 45 min on ice in the dark. As a negative control use cells that were treated with the same protocol but without antibodies or iso-type control antibodies instead of CXCR4-PE and CD49e-FITC.
6. Wash the cells twice with ice-cold flow cytometry buffer, finally resuspend the cells in sheath fluid or flow cytometry buffer, and measure the cells in a flow cytometer equipped with a 488 nm laser able to detect green (FITC) and yellow (PE) fluorescence. An example staining is presented in Fig. 2a.

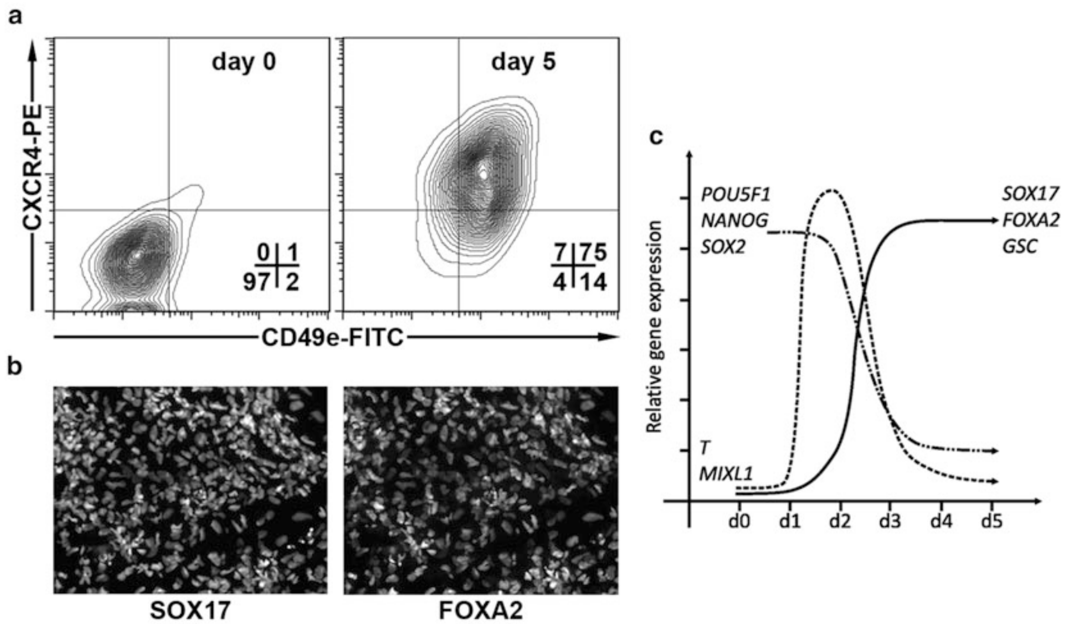

Fig. 2 Analysis of DE-specific markers by flow cytometry and immunofluorescence staining and a schematic gene expression profile of the expected gene expression changes. (**a**) Representative flow cytometry dot plot diagrams of the HUES8 human pluripotent stem cell line prior to (day 0) and after endoderm induction (day 5). The cells were stained for the surface markers CXCR4 and CD49e. The indicated numbers represent the percentage in each quadrant of the plot. (**b**) Fluorescence micrographs showing the protein expression of SOX17 and FOXA2 after 5 days of differentiation. (**c**) Schematic presentation of the expected gene expression changes during differentiation. Typically, pluripotency master regulators such as *POU5F1*, *SOX2*, and *NANOG* are drastically decreased once the medium conditions are changed to high activin A/Nodal and Wnt/beta catenin signaling. At the same time the transcription factors *T* and *MIXL1* present in the primitive streak are highly induced. Transcription factors of the endoderm gene regulatory network *SOX17*, *FOXA2*, and *GSC* peak in their expression during the last 48 h of endoderm differentiation. In parallel primitive streak markers are decreased in their expression

3.4 Analysis of Differentiation by Immunofluorescence

1. The number of DE-committed cells can be verified and quantified via nuclear co-localization of the core endoderm transcription factors FOXA2 and SOX17. An example of a typical SOX17/FOXA2 staining is presented in Fig. 2b.
2. Seed the cells on 4- or 8-well coverslips and differentiate the cells as outlined in Section 3.2.
3. Aspirate the medium and wash twice with PBS (*see* **Note 10**). Fix the cells with 4 % PFA in PBS for 10–20 min at 4 °C (*see* **Note 11**). Wash the cells three times with PBS and remove the excess of PBS after the last washing step.
4. Block the cells with 250–500 μl blocking buffer for 20 min at room temperature. Then aspirate the blocking buffer.
5. Add the incubation buffer containing the anti-SOX17 antibody diluted 1:250 and anti-FOXA2 antibody diluted 1:300. Incubate for 1–2 h at room temperature or overnight at 4 °C (*see* **Note 10**).
6. Aspirate the incubation buffer and wash three times for 5 min with PBS.
7. Add 500 μl incubation buffer containing the secondary antibodies directed against rabbit and goat each diluted 1:500. Incubate for 60 min at room temperature in the dark (*see* **Note 10**).
8. Aspirate the incubation buffer and wash three times for 5 min with PBS.
9. Mount the slides with immunoselect mounting medium. Dry the slides overnight at room temperature and store them until microscopical inspection at 4 °C in the dark.

3.5 RT-qPCR Gene Expression Analysis

3.5.1 RNA Extraction

1. Isolate total RNA from undifferentiated cells (starting population) and differentiated cells (day 5). To obtain a detailed insight into the kinetic of differentiation, it is recommended to sample every 24 h during differentiation so that the gene expression changes become detectable. A scheme summarizing the expected changes in gene expression is depicted in Fig. 2c.
2. Collect the cells as described in Section 3.2, points 3–5, but wash the cells once with PBS, spin them for 300 × *g*/3 min, and remove the supernatant. The cell pellet can be stored at −80 °C (or at −20 °C) until RNA isolation.
3. Add 0.5 ml Trizol/Trifast/Qiazol to the cell pellet and vigorously pipette the cell pellet up and down until all cells are lyzed. Incubate for 5 min at room temperature (*see* **Note 12**).
4. Add 100 ml chloroform and mix by pipetting up and down.
5. Centrifuge for 12,000 × *g*/15 min at 4 °C. The total RNA will remain in the upper nucleic aqueous phase, whereas the

intermediate phase contains the genomic DNA and the lower organic phase the denatured proteins.

6. Carefully transfer the supernatant into a fresh RNase-free reaction tube and mix with the same volume of 70 % ethanol.
7. Transfer the solution onto the column of the RNA extraction kit and centrifuge for 10,000 × *g*/30 s in a table centrifuge.
8. Follow the manufacturer's instructions beginning with the washing step.
9. Eluate the RNA and measure the concentration in a spectral photometer at 260/280 nm wavelength (*see* **Note 13**).

3.5.2 First-Strand cDNA Synthesis

1. The following description is based on the RevertAid first-strand cDNA synthesis kit by Thermo Fisher Scientific. However, the basic principle applies to most cDNA synthesis systems.
2. For one reaction mix 500 ng up to 5 μg total RNA with 0.2 μg random hexamer primer and add RNase-free water to a final volume of 12.5 μl. Incubate for 5 min at 65 °C.
3. Add 4 μl reverse transcriptase 5× reaction buffer, 0.5 μl RNase inhibitor, 2 μl 10 mM dNTP mix, and 1 μl RevertAid reverse transcriptase (200 U/μl). Mix gently and incubate for 10 min at 25 °C followed by 60 min at 42 °C.
4. Terminate the reaction for 5 min at 70 °C. The cDNA should be stored at −20 °C.

3.5.3 RT-qPCR Gene Expression Analysis

1. Dilute the cDNA sample to a final concentration of 5 ng/μl.
2. Set up a 15 μl PCR reaction for each gene in triplicate by adding the following components:
   (a) 7.5 μl 2× qPCR master mix.
   (b) 0.9 μl forward primer.
   (c) 0.9 μl reverse primer.
   (d) 2 μl cDNA.
   (e) 3.7 μl PCR-grade water.
3. Add a negative control to the plate (water blank) for each primer pair.
4. Cycling conditions: 95 °C for 10 min, followed by 40 cycles comprising 95 °C for 15 s and 60 °C for 60 s. Record the fluorescence after each cycle.
5. Perform a melting curve analysis: 95 °C for 15 s, cool down to 60 °C, and then continuous heating (heating rate of 0.02–0.25 °C/s) from 60 to 95 °C whilst recording the fluorescence values.
6. Check the melting curve data for the correct melting points of the amplicons. Calculate threshold values (CP or CT values) for

all RT-qPCR reactions and normalize the relative gene expression values by the efficiency-corrected delta-delta CT equation against the reference genes *G6PD*, *TBP*, and *TUBA1A* (*see* **Note 14**).

3.6 Purification of DE Cells by Magnetic Cell Separation

1. One well of a 6-well plate contains between 4 and 6 million cells after differentiation. The assay described here is for ten million cells. Thus, 2–3 wells provide enough material for purification by MACS.
2. Harvest DE-committed cells by trypsinization. Add 300 μl TE to each well and incubate for 3–5 min in the incubator.
3. Stop the reaction by adding 2 ml stopping medium.
4. Collect the cells, pass them through a cell strainer with a 40 μM mesh size, and count the cells.
5. Spin down ten million cells for 300 × *g*/3 min, aspirate the supernatant, and resuspend in 100 μl chilled PEB buffer.
6. Add 10 μl CXCR4-APC antibody (provided with the kit) and incubate for 20–30 min at 2–8 °C in the dark. Then, add 2 ml chilled PEB buffer. Spin down the cells for 300 × *g*/3 min, aspirate the supernatant, and resuspend in 80 μl chilled PEB buffer.
7. Meanwhile, insert an MS column and a collection tube into its magnetic stand. Rinse the MS column with 500 μl PEB buffer.
8. Add 20 μl anti-APC MicroBeads (provided with the kit) to the cells and incubate for 20–30 min at 2–8 °C in the dark. Then, add 2 ml chilled PEB buffer. Spin down the cells for 300 × *g*/3 min, aspirate the supernatant, and resuspend the cells in 500 μl PB buffer.
9. Add the labeled cells to the MS column and collect the flow-through (*see* **Note 15**).
10. Wash the column three times with 500 μl PEB buffer.
11. Remove the MS column from the magnetic stand and place it on a collection tube. Eluate the CXCR4-positive cells with 1 ml PEB buffer or culture medium and analyze the cells by flow cytometry. An example analysis of DE-committed cells after MACS sorting is presented in Fig. 3. For further differentiation of the endoderm population, the culture/differentiation medium should be adjusted to 10 μM ROCK inhibitor to enhance single-cell survival.

3.7 Purification by Fluorescence-Activated Cell Sorting

1. Harvest DE-committed cells by trypsinization. Add 300 μl TE to each well and incubate for 3–5 min in the incubator.
2. Stop the reaction by adding 2 ml stopping medium.

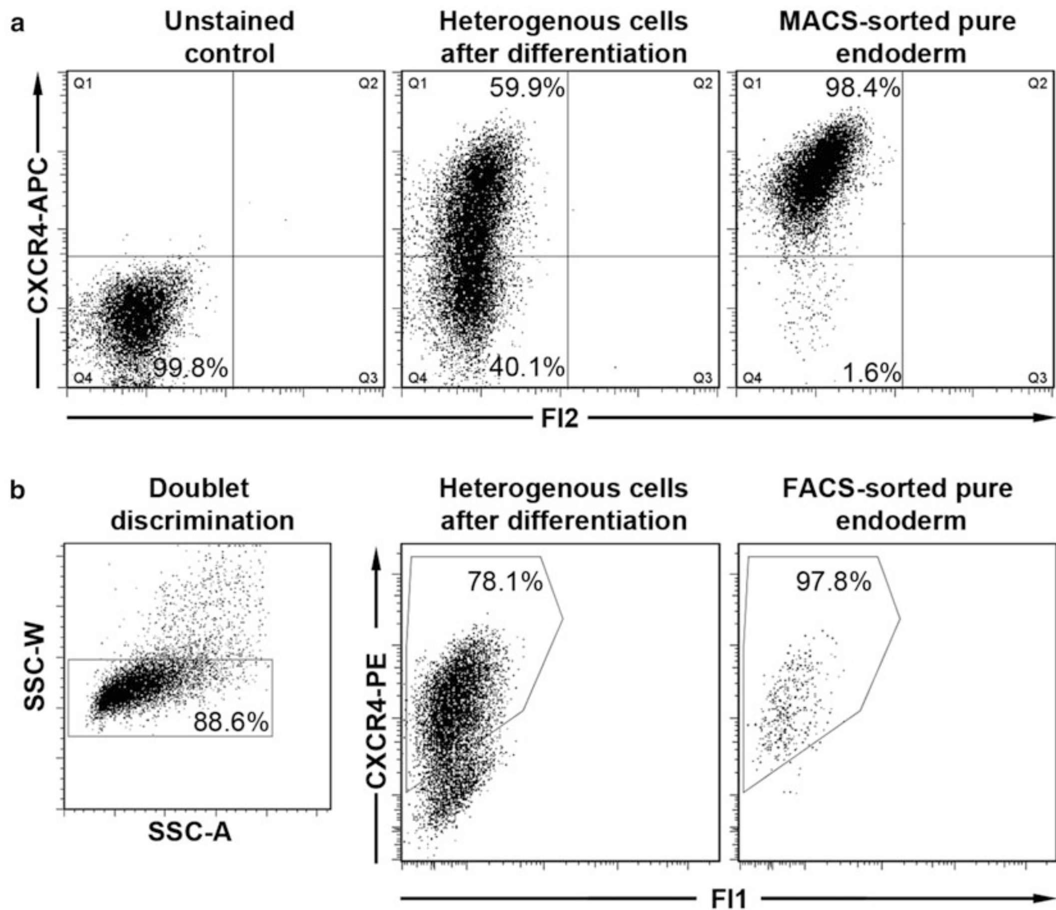

Fig. 3 Flow cytometry analysis of MACS- or FACS-sorted endoderm cells. Representative flow cytometry dot plot diagrams of the HES3 human pluripotent stem cell line after 5 days of endoderm differentiation are shown. (**a**) Depicted on the left is an unstained control, in the center a plot showing a heterogeneous population of CXCR4-APC-positive and -negative cells, and on the right MACS-sorted pure endoderm. (**b**) Depicted on the left is doublet discrimination by plotting the side scatter area against the side scatter width. The center shows a dot plot of CXCR4-PE-positive and -negative cells after differentiation. Shown on the right is a FACS reanalysis after the sorting procedure of the CXCR4-positive fraction, representing the purified endoderm cells

3. Collect the cells, pass them through a cell strainer with a 40 μM mesh size, and count the cells.
4. Spin down one million cells for 300 × *g*/3 min, aspirate the supernatant, and resuspend in 100 μl ice-cold sorting buffer.
5. Add 20 μl CXCR4-PE antibody (*see* **Note 9**) and incubate on ice for 45 min in the dark. Keep some unstained cells as a control.
6. Wash the cells twice with ice-cold flow cytometry buffer and finally resuspend the cells in 100 μl sorting buffer. Proceed with the cell sorting.

7. Sorting should be performed into liquid. Add culture medium or sorting buffer to the tubes prior to sorting.
8. Sort the cells with a cell sorter (e.g., BD FACSAria, Beckman Coulter MoFlo XDP) using a 70 μM nozzle. First gate the cells by plotting forward scatter area against side scatter area and then discriminate doublets by plotting the side scatter area against the side scatter width (Fig 3b). Finally gate on PE-positive cells and perform a reanalysis of the sorted cell population (*see* **Note 16**).
9. For further differentiation of the endoderm population, the culture/differentiation medium should be adjusted to 10 μM ROCK inhibitor to enhance single-cell survival.

4 Notes

1. The hESC-qualified Matrigel should be stored in aliquots at −80 °C following the manufacturer's instructions. To coat tissue culture dishes, plates, or flasks, Matrigel should be slowly thawed on ice. One aliquot is then resuspended in ice-cold knockout DMEM/F-12 and subsequently tissue culture material can be coated for 60 min at room temperature or alternatively 15 min in the tissue culture incubator at 37 °C. After the coating, the supernatant still contains matrix proteins that have not yet polymerized; thus, Matrigel may be reused. For that purpose used Matrigel should be stored at 4 °C for up to 7 days.
2. The complete mTeSR™1 culture medium has a short shelf life of only 14 days at 4 °C. The medium should be stored in aliquots at −20 °C and thawed in inappropriate amounts. The volume required for cultivation on that day should be warmed up separately from the thawed working medium, which should always be stored at 4 °C.
3. The nonenzymatic passaging solution can be purchased commercially or prepared at very low costs and efforts. The osmolality should be adjusted to 570 mOsm/kg. Lower values result in an increased colony detachment into single cells. Higher osmolalities will unlikely have a significant effect (details published in (15)).
4. Optimal incubation times should be determined individually for each cell line. Highly confluent cultures might need a longer incubation time.
5. The optimal passaging ratio is dependent on the cell line and the reattachment rate. It should be determined individually for each cell line. Addition of ROCK inhibitor Y-27632 may result in a higher split ratio. However, the passaged PSCs we

keep in our laboratory showed a better morphology without Y-27632.

6. The colonies should detach as single cells. If necessary prolong incubation time and pass the cells through a 40 μM cell strainer to obtain a solution free of cell clusters.
7. 100,000 cells/cm^2 were found to be optimal for definitive endoderm induction for all tested human PSCs. However, if the reattachment rate of the human pluripotent cell line after single-cell dissociation is low, higher numbers should be used. Generally a minimum cell density is required to ensure an efficient differentiation that should be separately determined for each human PSC line.
8. The optimal concentrations of CHIR-99021 and activin A should be determined for each human PSC line. The concentrations denoted here worked for all tested human PSC lines and induced high numbers of DE-committed cells (9). However, it might be worthwhile to define a lower threshold concentration especially for the recombinant protein activin A, which is expensive and puts a huge cost pressure on the laboratory.
9. The antibody volume for a satisfactory immunolabeling can vary between different lots. It is recommended to test every new lot in a dilution series to specify the required amounts.
10. The incubations with antibodies and the washing steps should be performed without shaking as our cells sometimes detached from the slides as a result of the shaking.
11. Fixation of tissue culture cells with PFA usually takes no more than 10 min, unless the cells are grown in larger tissue-like clusters. We found that extended incubation times in PFA decreased or destroyed the antigens for the FOXA2 antibody. Fixed cells can be stored at 4 °C in PBS until the staining is performed. Prior to long-term storage, the PFA should be removed by washing them twice with PBS. It is recommended to add penicillin/streptomycin or other antibiotics to the PBS and to seal the plates with parafilm to reduce evaporation of the liquid.
12. The phenol:chloroform extraction is sufficiently efficient to remove genomic DNA during the RNA purification. Optionally, an on-column DNase I treatment might be performed to further reduce the presence of genomic DNA in the nucleic acid fraction. It is highly recommended to remove genomic DNA, either by phenol:chloroform extraction or DNase I treatment since traces of genomic DNA carried over into downstream PCR applications might lead to false-positive results.

13. It is recommended to analyze the quality of the RNA in a denaturing agarose gel prior to further downstream applications. Degraded RNA has a distinct influence on the validity and reliability of RT-qPCR results (21). The presence and integrity of the 18S and 28S ribosomal RNA bands should be checked in an agarose gel or, if possible, analyzed by microfluidic capillary electrophoresis (e.g., in a Agilent 2100 Bioanalyzer or Biorad Experion). Absence or disintegration of one ribosomal RNA band, especially the upper 28S band, indicates the presence of RNases. These RNases will certainly cause partial or full degradation of messenger RNA. In a perfect total RNA sample the upper 28S band appears twice as intense as the lower 18S band without any molecular smear.
14. There is a remarkable lack of consensus on how to design, perform, and analyze RT-qPCR assays. This might lead to exaggerated or even false-positive data. Bustin and coworkers addressed this issue and proposed a certain degree of conceptual considerations, sample handling procedures, and normalization strategies for qPCR/RT-qPCR termed MIQE guidelines (22). It is highly recommended to gather crucial information about qPCR normalization strategies from the literature (23–25). Especially the stability of the used normalizer genes should be carefully addressed.
15. The efficacy of a single-column MACS sorting might be disappointing so that the flow-through of the first column may be passed over a second, fresh MS column. Treat both columns equally and pool the eluates to increase the number of CXCR4-positive sorted cells.
16. The sorting procedure will produce the best result if the cells are in a homogenous single-cell suspension. The EDTA concentration in the sorting buffer may be increased to 5 mM EDTA to inhibit clumping. The sorting procedure itself is stressful to the cells so that the viability is lower compared to MACS-purified cells. If very low viability is observed, it could be helpful to use a larger nozzle diameter such as 100 μM and to lower the sorting speed. To increase the purity of the endoderm fraction, cell clusters should be removed by filtering through a cell strainer with a 40 μM mesh size. Doublet discrimination should be carried out by appropriate gating.

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Definitive Endoderm Differentiation of Human Embryonic Stem Cells Combined with Selective Elimination of Undifferentiated Cells by Methionine Deprivation

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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 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 days (d) 8 to (d) 10. 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

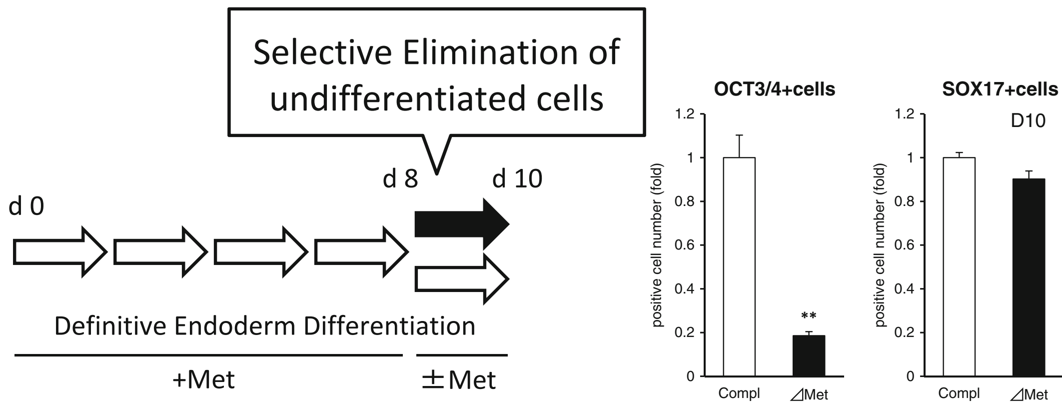

Fig. 1 Human ESCs (khES1) were induced into definitive endoderm through 10-day differentiation, with or without methionine deprivation from differentiation days (d) 8 to (d) 10. Methionine deprivation resulted in eliminating undifferentiated cells (marked by *OCT3/4* expression) without reducing endoderm cells (marked by *SOX17* expression). *Error bars* represent SEM ($n = 3$). Significant differences were determined by Student's *t* test; $^{**}p < 0.01$

metabolic difference, we developed a protocol to eliminate the remaining undifferentiated cells [11].

This protocol is based on our previous culture system (co-culture with M15 feeder cells in RPMI supplemented with 100 ng/mL activin A for 10 days) [4] with a slight modification in using methionine-deprived medium from d8 to d10. A protocol without using M15 cells is also described. *See* Fig. 1 for an overview.

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	2 mL

   (Final concentration: 10 μg per mL).
9. Human ESC medium.

Reproff (ReproCELL, RCHEMD004)	500 mL
PS (Nacalai Tesque, 26252-94) (*see* **Note 2**)	5 mL

10. Supplements for human ESC 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 ESC 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 and Technology Institute, a custom-made medium)	500 mL

(continued)

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)

(a) *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.

(b) *Passage of M15 cells*

1. At full confluent, 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.

(c) *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 $180 \times g$ 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-well 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 °C freezer and put into 37 °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 (Subheading 3.2).
6. Incubate at 37 °C under 5 % CO_2.
7. On the next day, MMC-M15 cells are ready to be used as feeders for human ESC differentiation.

3.4 Plating and Differentiation of Human ESCs (See Note 5)

1. Remove human ESC 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 × *g* for 5 min.
8. Resuspend the pellet with human ESC medium supplemented with 10 μM Y27632 to the concentration at 1×10^5 cells/mL.
9. Remove EF medium from the MMC-M15 cell plates (Subheading 3.3) and add 100 μL fresh human ESC medium with 10 μM Y27632 into MMC-M15 cell plates.
10. Add 100 μL of cell suspension into MMC-M15 96-well plate pre-added with 100 μL of human ESC medium.
11. Incubate at 37 °C under 5 % CO_2.
12. On the next day, remove human ESC medium.
13. Rinse with PBS.
14. Change medium with fresh endoderm differentiation medium 1 supplemented with both activin and B27 at days 0, 2, 4, and 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 ESC 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 × *g* for 5 min.
8. Resuspend the pellet with human ESC 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 ESC 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 ESC medium.
11. Incubate at 37 °C under 5 % CO_2.
12. On the next day, remove human ESC medium.
13. Rinse with PBS.
14. Change medium with fresh endoderm differentiation medium 1 supplemented with both activin and B27 at days 0, 2, 4, and 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**).

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, autoclave, and store at room temperature.
5. One day before plating, human ESCs are cultured in human ESC medium supplemented with 10 μM Y27632. At 80 % confluent, 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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Selective Differentiation into Hematopoietic and Cardiac Cells from Pluripotent Stem Cells Based on the Expression of Cell Surface Markers

Atsumasa Okada, Katsuhisa Tashiro, Tomoko Yamaguchi, and Kenji Kawabata

Abstract

Flk1-expressing (+) mesodermal cells are useful source for the generation of hematopoietic cells and cardiomyocytes from pluripotent stem cells (PSCs). However, they have been reported as a heterogenous population that includes hematopoietic and cardiac progenitors. Therefore, to provide a method for a highly efficient production of hematopoietic cells and cardiomyocytes, cell surface markers are often used for separating these progenitors in Flk1$^+$ cells. Our recent study has shown that the expression of coxsackievirus and adenovirus receptor (CAR), a tight junction component molecule, could divide mouse and human PSC- and mouse embryo-derived Flk1$^+$ cells into Flk1$^+$CAR$^-$ and Flk1$^+$CAR$^+$ cells. Flk1$^+$CAR$^-$ and Flk1$^+$CAR$^+$ cells efficiently differentiated into hematopoietic cells and cardiomyocytes, respectively. These results indicate that CAR is a novel cell surface marker for separating PSC-derived Flk1$^+$ mesodermal cells into hematopoietic and cardiac progenitors. We herein describe a differentiation method from PSCs into hematopoietic cells and cardiomyocytes based on CAR expression.

Keywords: Mesoderm, Differentiation, CAR, Hematopoietic cells, Cardiomyocytes, Flk1

1 Introduction

During mouse embryogenesis, Flk1-expressing (+) cells are well known as mesodermal cells, which give rise to hematopoietic cells and cardiomyocytes (1, 2). Flk1$^+$ cells can be differentiated from pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), and produce hematopoietic cells and cardiomyocytes (2–6). Therefore, PSCs-derived Flk1$^+$ cells are a useful source for the generation of hematopoietic cells and cardiomyocytes. On the other hand, Flk1$^+$ cells have been reported to be a heterogenous population containing at least hematopoietic and cardiac progenitor cells (2), suggesting that separation of Flk1$^+$ cells into distinct mesodermal progenitors would allow us to establish a method for a highly efficient production of hematopoietic cells and cardiomyocytes.

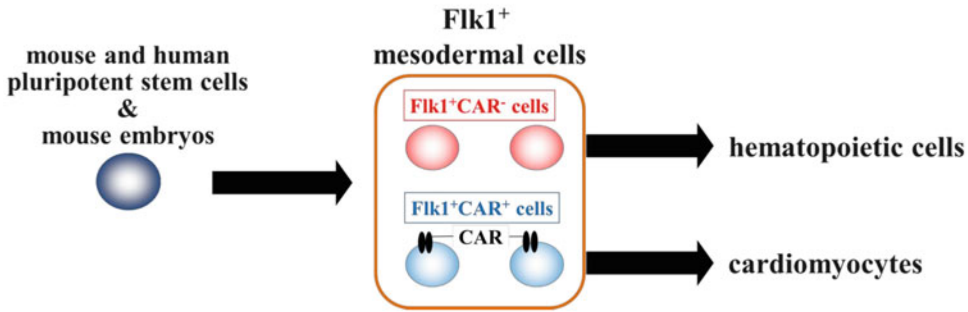


Fig. 1 Differentiation potentials of two Flk1$^+$ subsets. Mouse and human PSC- and mouse embryo-derived Flk1$^+$ mesodermal cells could be separated into two populations (Flk1$^+$CAR$^-$ cells and Flk1$^+$CAR$^+$ cells). Flk1$^+$CAR$^-$ cells and Flk1$^+$CAR$^+$ cells have the potential to differentiate into hematopoietic cells and cardiomyocytes, respectively

In our recent study, we have focused on coxsackievirus and adenovirus receptor (CAR), which is originally identified as a cell surface receptor for coxsackie B viruses and C-type adenoviruses and is also known as a tight junction component molecule (7, 8). It has been reported that the CAR expression is low or undetectable in hematopoietic cells, and is high in the heart (8–12), raising a possibility that Flk1$^+$ cells could be separated into hematopoietic and cardiac progenitor cells based on CAR expression. To test this possibility, we examined CAR expression in the mesodermal differentiation of PSCs, and found that mouse PSC- and embryo-derived Flk1$^+$ cells could be divided into two types of cells; CAR$^+$ and CAR$^-$ cells (13). Flk1$^+$CAR$^+$ cells have the cardiac differentiation potential, and Flk1$^+$CAR$^-$ cells efficiently differentiated into hematopoietic cells (13). In addition, CAR$^+$ and CAR$^-$ cells were also identified in human PSCs-derived KDR$^+$ (human counterpart to Flk1) mesodermal, and KDR$^+$CAR$^+$ and KDR$^+$CAR$^-$ cells have cardiac and hematopoietic differentiation potential, respectively (13). Therefore, the tight junction molecule CAR would be a useful marker for separating mouse and human PSC- and embryo-derived Flk1$^+$ mesodermal cells into hematopoietic and cardiac progenitors (Fig. 1). In this chapter, we provide a novel method for hematopoietic and cardiac differentiation with PSCs and mouse embryos.

2 Materials

2.1 Mouse ESCs/iPSCs

2.1.1 Cell Lines

1. Mouse ES cell line, BRC6 (Riken Bioresource Center).
2. Mouse iPS cell line, 38C2 (a gift from Dr. Shinya Yamanaka, Kyoto University) (14).

2.1.2 Cell Maintenance

1. ESC-Sure mESC Complete Medium (Applied StemCell, Inc.).
2. 1,000× Leukemia inhibitory factor (LIF; Wako).
3. 2-Mercaptoethanol (2-ME; Nacalai Tesque).
4. Culture medium (mESC/iPSC medium): ESC-Sure mESC Complete Medium supplemented with LIF (1×) and 2-ME (100 μM).
5. Phosphate-Buffered-Saline without Ca^{2+} and Mg^{2+} (PBS).
6. 0.25 % trypsin–EDTA solution (Life Technologies).
7. StemSure 0.1 w/v% gelatin solution (Gelatin; Wako).
8. 60-mm cell culture dishes (Nunc).
9. Mitomycin C-treated mouse embryonic fibroblasts (MEFs; Millipore).

2.1.3 Embryoid Body Formation

1. Dulbecco's modified Eagle's medium (DMEM; Wako).
2. Fetal Bovine Serum (FBS; Life Technologies).
3. 100× Non-Essential Amino Acids (NEAA; Life Technologies).
4. 100 mM L-glutamine (Life Technologies).
5. 100× Nucleoside (Millipore).
6. 2-ME.
7. Penicillin–Streptomycin (Pen-Strep; Life Technologies).
8. Differentiation medium (mEB medium): DMEM containing FBS at 15 % supplemented with NEAA (1×), L-glutamine (2 mM), nucleoside (1×), 2-ME (100 μM), and Pen-Strep.
9. Lipidure-coat 96-well plates (Thermo Fisher Scientific).

2.1.4 Cell Sorting

1. PBS.
2. FBS.
3. FACS buffer: PBS containing FBS at 2 %.
4. 0.25 % trypsin–EDTA solution.
5. 70 μm cell strainer (BD Bioscience).
6. Antibodies (*see* Tables 1 and 2).

2.1.5 OP9 Stromal Cell Maintenance

1. OP9 stromal cells (Riken Bioresource Center).
2. α-Minimum essential medium (αMEM; Sigma).
3. FBS.
4. L-glutamine.
5. NEAA.
6. Pen-Strep.

Table 1
List of primary antibodies used in this study

Species	Antigen	Clone	Host	Conjugated molecules	Application	Company
Mouse	CAR	4C9	Rat	–	FACS	–
	Flk1	Avas12a1	Rat	Biotin	FACS	e-Bioscience
	CD45	30-F11	Rat	FITC	FACS	e-Bioscience
	Ter119	TER-119	Rat	PE	FACS	e-Bioscience
Human	CAR	RmcB	Mouse	–	FACS	Millipore
	KDR	89106	Mouse	PE	FACS	R&D Systems
	CD45	HI30	Mouse	PECy7	FACS	BD Bioscience
	CD144	16B1	Mouse	PE	FACS	e-Bioscience
Mouse and human	cTNT	11–13	Mouse	–	Immuno staining	Thermo Scientific

Table 2
List of secondary antibodies used in this study

Secondary antibodies	Application	Company
Streptavidin-Brilliant Violet 421	FACS	BioLegend
Rat IgG-DyLight 649	FACS	BioLegend
Mouse IgG-Brilliant Violet 421	FACS	BioLegend
Mouse IgG-Alexa Fluor 594	Immunostaining	Life Technologies

7. Culture medium (OP9 medium): αMEM containing FBS at 20 % supplemented with L-glutamine (2 mM), NEAA (1×), and Pen-Strep.
8. PBS.
9. 0.25 % trypsin–EDTA solution.
10. 100-mm cell culture dishes (Nunc).

2.1.6 Hematopoietic Differentiation from Mouse ESC/iPSC-Derived Cells

1. OP9 medium.
2. Mouse stem cell factor (mSCF; Peprotech).
3. Human Flt3-ligand (hFlt3-L; Peprotech).
4. Mouse thrombopoietin (mTPO; Peprotech).
5. Mouse interleukin-3 (mIL-3; R&D Systems).
6. Human interleukin-6 (hIL-6; Peprotech).
7. 2-ME.

Table 3
List of primers used in this study

Species	Gene	Forward (5′–3′)	Reverse (5′–3′)
Mouse	*Gapdh*	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
	Scl	AACAACAACCGGGTGAAGAG	GGGAAAGCACGTCCTGTAGA
	Runx1	CCAGGTAGCGAGATTCAACGA	CAACTTGTGGCGGATTTGTAAA
	Gata-1	TTGTGAGGCCAGAGAGTGTG	TTCCTCGTCTGGATTCCATC
	Gata-2	TAAGCAGAGAAGCAAGGCTCGC	ACAGGCATTGCACAGGTAGTGG
	Fli-1	CCAACGAACGGAGAGTCATT	ATTCCTTGCCATCCATGTTC
	Tbx5	CTACCCCGCGCCCACTCTCAT	TGCGGTCGGGGTCCAACACT
	Mesp1	GCTCGGTCCCCGTTTAAGC	ACGATGGGTCCCACGATTCT
	Gata-4	CCACGGGCCCTCCATCCAT	GGCCCCCACGTCCCAAGTC
	Myl4	AAGAAACCCGAGCCTAAGAAGG	TGGGTCAAAGGCAGAGTCCT
	cTNT	GCGGAAGAGTGGGAAGAGACAGAC	GCACGGGGCAAGGACACAAG
	αMHC	GCTGGGCTCCCTGGACATTGAC	CCTGGGCCTGGATTCTGGTGAT
Human	*Gapdh*	GGTGGTCTCCTCTGACTTCAACA	GTGGTCGTTGAGGGCAATG
	Runx1	TGGCTGGCAATGATGAAAAC	CACTTCGACCGACAAACCTGA
	Scl	AGCCGGATGCCTTCCCTAT	GGGACCATCAGTAATCTCCATCT
	Gata-2	GCAACCCCTACTATGCCAACC	CAGTGGCGTCTTGGAGAAG
	Isl1	TTGTACGGGATCAAATGCGCCAAG	AGGCCACACAGCGGAAACA
	Tbx5	AAATGAAACCCAGCATAGGAGCTGGC	ACACTCAGCCTCACATCTTACCCT
	cTNT	TTCACCAAAGATCTGCTCCTCGCT	TTATTACTGGTGTGGAGTGGGTGTGG

8. Differentiation medium: OP9 medium supplemented with mSCF (50 ng/mL), hFlt3-L (50 ng/mL, Peprotech), mTPO (10 ng/mL), mIL-3 (5 ng/mL), hIL-6 (5 ng/mL), and 2-ME (50 μM).
9. 24-well culture plates (Nunc).

2.1.7 Cardiac Differentiation from Mouse ESC/iPSC-Derived Cells

1. OP9 medium.
2. 2-ME.
3. Differentiation medium: OP9 medium supplemented with 2-ME (50 μM).
4. 24-well, 48-well culture plates (Nunc).

2.2 Mouse Embryos

2.2.1 Cell Sorting

1. Cell dissociation buffer, enzyme-free, PBS (Life Technologies).

Other materials are described in Section 2.1.4.

2.2.2 OP9 Stromal Cell Maintenance

See Section 2.1.5.

2.2.3 Hematopoietic Differentiation from Mouse Embryo-Derived Cells

See Section 2.1.6.

2.2.4 Cardiac Differentiation from Mouse Embryo-Derived Cells

See Section 2.1.7.

2.3 Human ESCs/iPSCs

2.3.1 Cell Lines

1. Human ES cell line, KhES-3 (provided by Dr. Norio Nakatsuji, Kyoto University) (15).
2. Human iPS cell line, 201B7 (provided by Dr. Shinya Yamanaka, Kyoto University) (16).

2.3.2 Cell Maintenance

1. Repro Stem medium (ReproCELL, Tokyo, Japan).
2. Human fibroblast growth factor-2 (hFGF2, Katayama Kagaku Kogyo).
3. Culture medium (hESC/iPSC medium): Repro Stem medium supplemented with hFGF2 (5 ng/mL).
4. StemSure 0.1 w/v% gelatin solution (Gelatin; Wako).
5. 100-mm cell culture dishes.
6. Mitomycin C-treated mouse MEFs.

2.3.3 Embryoid Body Formation

1. StemPro-34 SFM (Life Technologies).
2. StemPro-34 nutrient supplement (Life Technologies).
3. Ascorbic acid (AA, Sigma).
4. Monothioglycerol (MTG, Sigma).
5. Rock inhibitor (Y-27632; Wako).
6. Human bone morphogenetic protein 4 (hBMP4; R&D Systems).
7. Human Activin-A (hActivin-A; R&D Systems).
8. hFGF2.
9. Human vascular endothelial growth factor (hVEGF, Peprotech, Rocky Hill, NJ).
10. Human Dickkopf1 (hDKK1, R&D Systems).
11. L-Glutamine.
12. Pen-Strep.
13. Basal medium: StemPro-34 SFM containing StemPro-34 nutrient supplement, L-glutamine (2 mM), and Pen-Strep.
14. Differentiation medium 1 (hEB-1 medium): basal medium supplemented with Y-27632 (10 μM).

15. Differentiation medium 2 (hEB-2 medium): basal medium supplemented with hBMP4 (2 ng/mL), Y27632 (10 μM), AA (50 μg/mL), and MTG (450 μM).
16. Differentiation medium 3 (hEB-3 medium): basal medium supplemented with hBMP4 (10 ng/mL), hActivin-A (6 ng/mL), hFGF2 (5 ng/mL), AA (50 μg/mL), and MTG (450 μM).
17. Differentiation medium 4 (hEB-4 medium): basal medium supplemented with hVEGF (10 ng/mL), hDkk1 (10 ng/mL), AA (50 μg/mL), and MTG (450 μM).
18. Dispase (Roche).
19. Minimum Essential Medium Eagle (MEM; Sigma).
20. Dispase solution: MEM containing dispase (0.1 mg/mL).
21. Gelatin.
22. 100-mm cell culture dishes.
23. 100-mm petri dishes (AS ONE).

2.3.4 Cell Sorting

See Section 2.1.4.

2.3.5 OP9 Stromal Cell Maintenance

See Section 2.1.5.

2.3.6 Hematopoietic Differentiation from Human ESC/iPSC-Derived Cells

1. OP9 medium.
2. mSCF.
3. hFlt3-L.
4. mTPO.
5. Human interleukin-3 (hIL-3; Peprotech).
6. hIL-6.
7. AA.
8. MTG.
9. 2-ME.
10. Differentation medium: OP9 medium supplemented with mSCF (100 ng/mL), hFlt3-L (100 ng/mL), mTPO (10 ng/mL), hIL-3 (10 ng/mL), hIL-6 (10 ng/mL), AA (50 μg/mL), MTG (450 μM), and 2-ME (50 μM).
11. 24-well culture plates.

2.3.7 Cardiac Differentiation from Human ESC/iPSC-Derived Cells

1. Basal medium (*see* Section 2.3.3, **Step 13**).
2. AA.
3. MTG.
4. hVEGF.

5. hFGF2.
6. Y-27632.
7. Differentiation medium-1: basal medium supplemented with hVEGF (10 ng/mL), hFGF2 (10 ng/mL), AA (50 μg/mL), and MTG (450 μM).
8. Differentiation medium-2: Differentiation medium-1 supplemented with Y-27632 (10 μM).
9. Gelatin.
10. 96-well culture plates (Nunc).

3 Methods

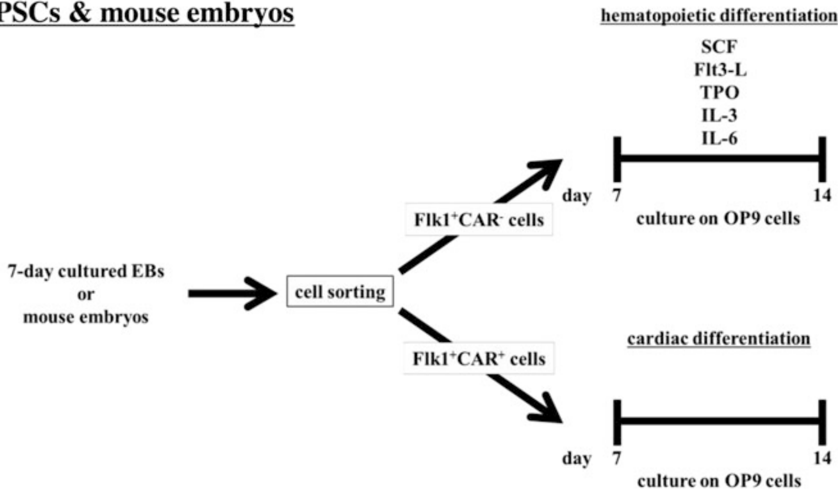
3.1 Differentiation of Mouse ESCs/iPSCs to Hematopoietic and Cardiac Cells (see Fig. 2)

3.1.1 Mouse ESC/iPSC Culture

1. Prepare MEF feeder layer on gelatin-coated 60 mm culture dishes.
2. Culture mouse ESCs (BRC6) and iPSCs (38C2) in 3–4 mL of mESC/iPSC medium on MEF feeder layer.
3. Change entire medium daily.
4. Passage the cells to fresh MEF feeder layer every 2–3 days as described below.
5. Aspirate the culture medium, wash with PBS twice, and then add 0.5 mL of 0.25 % trypsin–EDTA solution.
6. Incubate the culture at 37 °C for 3–5 min.
7. Gently dissociate the cell aggregates into single cells by pipetting.
8. Add 1.0 mL of mESC/iPSC medium and transfer the dissociated cells into 15 mL tube.
9. Centrifuge at 367 × *g* for 5 min at room temperature.
10. Resuspend the cells in 0.5 mL of mESC/iPSC medium.
11. Seed a part of the cell suspension (1:10–20) on fresh MEFs feeder layers.

3.1.2 Embryoid Body Formation

1. Harvest mouse ESCs/iPSCs and spin down as described above (*see* Section 3.1.1, **Steps 5–9**).
2. Aspirate the supernatant, suspend in 5 mL of mEB medium, and then plate on 60 mm cell culture dish.
3. Incubate the cells at 37 °C and 5 % CO_2 for 30 min to remove MEFs.
4. Transfer the suspension into 15 mL tube.
5. Centrifuge at 367 × *g* for 5 min at room temperature.
6. Resuspend the cells in 2–5 mL of mEB medium.



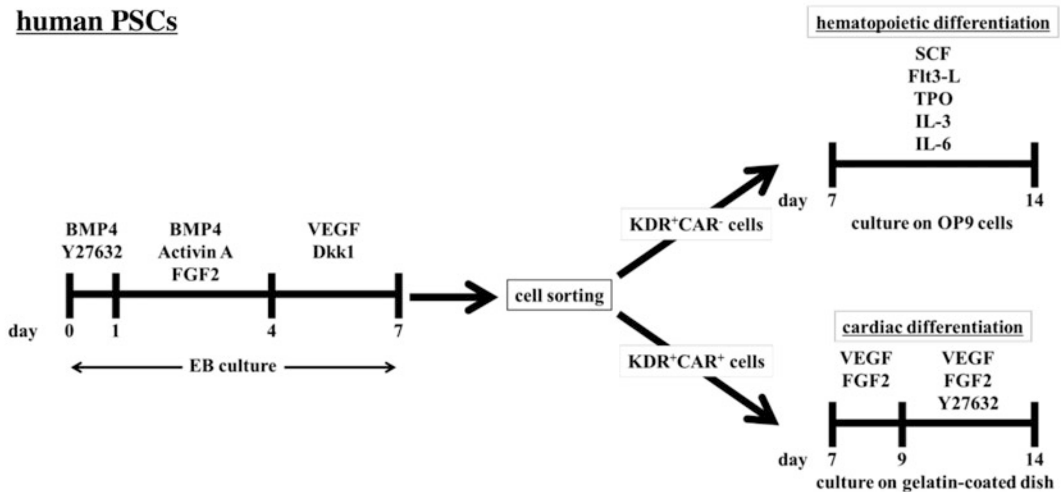


Fig. 2 Selective differentiation into hematopoietic and cardiac cells from pluripotent stem cells. Experimental protocols for hematopoietic and cardiac cells differentiation from mouse and human PSCs- and mouse embryo-derived cells are shown

7. Plate the dissociated cells on Lipidure-coated 96-well plates (BRC6 cells: 3.0×10^3 cells/well, 38C2 cells: 1.0×10^3 cells/well) with mEB medium (100 μL/well). We usually prepare five plates for cell sorting.
8. On day 2, add 100 μL of fresh mEB medium per well.
9. On day 5, change half of the medium (100 μL/well).
10. On day 7, harvest EBs and prepare them for cell sorting (*see* **Note 1**).

3.1.3 Cell Sorting

1. Transfer 7 day-cultured EBs into 50 mL tube.
2. Aspirate the culture medium, wash with PBS, and then add 1.0 mL of 0.25 % trypsin–EDTA solution.
3. Incubate the culture at 37 °C for 3–5 min.
4. Gently dissociate the EBs into single cells by pipetting.
5. Add 9.0 mL of FACS buffer (*see* Section 2.1.4, 3), gently mix the cell suspension by pipetting, and then pass through 70 μm cell strainer.
6. Transfer the suspension into 15 mL tube.
7. Centrifuge at 367 × *g* for 5 min at room temperature.
8. Resuspend the cells in 0.5 mL of FACS buffer.
9. Add an appropriate concentration of rat anti-mouse CAR antibody and incubate for 30 min at 4 °C.
10. Wash the cells with FACS buffer twice.
11. Resuspend the cells in 0.5 mL of FACS buffer.
12. Add an appropriate concentration of DyLight 649-conjugated anti-rat IgG antibody and incubate for 20 min at 4 °C.
13. Wash the cells with FACS buffer three times.
14. Resuspend the cells in 0.5 mL of FACS buffer.
15. Add an appropriate concentration of biotin-conjugated rat anti-mouse Flk1 antibody and incubate for 30 min at 4 °C.
16. Wash the cells with FACS buffer twice.
17. Resuspend the cells in 0.5 mL of FACS buffer.
18. Add an appropriate concentration of Brilliant Violet-conjugated streptavidin and incubate for 20 min at 4 °C.
19. Wash the cells with FACS buffer twice.
20. Resuspend the cells in 5–10 mL of FACS buffer and then pass through 70 μm cell strainer just before cell sorting.
21. Sorting two $Flk1^+$ subsets ($Flk1^+CAR^-$ cells and $Flk1^+CAR^+$ cells) with FACS Aria or FACS AriaII (BD Bioscience).

3.1.4 Hematopoietic Differentiation from Mouse ESC/iPSC-Derived Cells

1. A day before sorting, prepare 24-well plates with 80–90 % confluent monolayer of OP9 cells cultured in OP9 medium (*see* Section 2.1.5, 7).
2. Seed the FACS-sorted Flk1+CAR– cells on feeder layers of OP9 cells with differentiation medium (*see* Section 2.1.6, 8) at a density of 1.0–5.0 × 10^4 cells/well.
3. After 4–7 days in culture, confirm the hematopoietic differentiation of the FACS sorted $Flk1^+CAR^-$ cells by FACS and RT-PCR analyses (*see* **Note 2**).

3.1.5 Cardiac Differentiation from Mouse ESC/iPSC-Derived Cells

1. A day before sorting, prepare 24-well or 48-well plates with 80–90 % confluent monolayer of OP9 cells cultured in OP9 medium.
2. Seed FACS-sorted Flk1+CAR+ cells on feeder layers of OP9 cells with differentiation medium (*see* Section 2.1.7, 3, 500 μL/well) at a density of 1–5 × 10^4 cells/well (24-well plate) or 1–10 × 10^3 cells/well (48-well plate).
3. After 3–4 days in culture, add 500 μL of fresh differentiation medium.
4. On day 7, confirm the cardiac differentiation of FACS-sorted Flk1+CAR+ cells by immunocytochemical and RT-PCR analyses (*see* **Note 3**).

3.2 Differentiation of Mouse Embryo-Derived Cells to Hematopoietic and Cardiac Cells

3.2.1 Dissociation of Mouse Embryos

1. Prepare E8.5 mouse embryos.
2. Transfer into 1.5 mL tube, wash with PBS, and then add 0.3–0.5 mL of cell dissociation buffer.
3. Incubate the culture at 37 °C for 15 min.
4. Gently dissociate into single cells by pipetting.
5. Add 0.5–1.0 mL of FACS buffer and then mix the cell suspension gently by pipetting.
6. Centrifuge at 2,400 × *g* for 5 min at room temperature.
7. Resuspend the cells in 0.5 mL of FACS buffer.

3.2.2 Cell Sorting

See Section 3.1.3, **Steps 9–22**.

3.2.3 Hematopoietic Differentiation from Mouse Embryo-Derived Cells

See Section 3.1.4.

3.2.4 Cardiac Differentiation from Mouse Embryo-Derived Cells

See Section 3.1.5.

3.3 Differentiation of Human ESCs/iPSCs to Hematopoietic and Cardiac Cells

3.3.1 Human ESC/iPSC Culture

1. Prepare MEF feeder layers on gelatin-coated 100 mm culture dishes.
2. Culture human ESCs (KhES-3) and iPSCs (201B7) in 8–10 mL of the culture medium (*see* Section 2.3.2, **Step 3**) on MEF feeder layer.
3. Change entire medium daily.
4. Passage the cells to fresh MEF feeder layers every 5–7 days either with dispase or by colony picking.

3.3.2 Embryoid Body Formation

1. Prepare two or three dishes of 5–7 day-cultured human ESCs/iPSCs.
2. Replace the culture medium to 10 mL of basal medium (*see* Section 2.3.3, **Step 13**).
3. Incubate at 37 °C and 5 % CO_2 for 30 min.
4. Aspirate the basal medium and then add 1–2 mL of dispase solution (*see* Section 2.3.3, **Step 13**).
5. Incubate at 37 °C and 5 % CO2 for 5–10 min.
6. Aspirate the dispase solution, and then add 10 mL of basal medium.
7. Harvest the small clumps of hESC/iPSC colonies into 15 mL tube.
8. Centrifuge at 15 × *g* for 2 min at room temperature.

11. Carefully resuspend the clumps in 10 mL of hEB-1 medium (*see* Section 2.3.2, **Step 14**), and then plate on gelatin-coated 100-mm cell culture dish.
12. Incubate at 37 °C and 5 % CO_2 for 2 h to remove MEFs.
13. Harvest the clumps into 15 mL tube.
14. Centrifuge at 15 × *g* for 2 min at room temperature.
15. Resuspend the clumps in 10 mL of hEB-2 medium (*see* Section 2.3.2, **Step 15**) and then plate on a 100 mm petri dish.
16. On day 1, change the culture medium; harvest the clump-derived EBs into 15 mL tube, centrifuge at 15 × *g* for 2 min at room temperature, carefully and gently resuspend the EBs in 10 mL of hEB-3 medium (*see* Section 2.3.2, **Step 15**), and then plate on fresh 100 mm petri dish.
17. On day 4, change the culture medium to 10 mL of hEB-4 medium (*see* Section 2.3.2, **Step 16**) as described above.
18. On day 7, harvest EBs and prepare them for cell sorting (*see* **Note 4**).

3.3.3 Cell Sorting

1. Transfer 7 day-cultured EBs into 15 mL tube.
2. Aspirate the culture medium, wash with PBS, and then add 1.0 mL of 0.25 % trypsin–EDTA solution.
3. Incubate the culture at 37 °C for 10 min.
4. Gently dissociate the EBs into single cells by pipetting.
5. Add 9.0 mL of FACS buffer and then gently mix the cell suspension by pipetting.
6. Centrifuge at 367 × *g* for 5 min at room temperature.
7. Resuspend the cells in 0.5 mL of FACS buffer.
8. Add an appropriate concentration of mouse anti-human CAR antibody and incubate for 30 min at 4 °C.

9. Wash the cell solution with FACS buffer twice.
10. Resuspend the cells in 0.5 mL of FACS buffer.
11. Add an appropriate concentration of BV421-conjugated anti-mouse IgG antibody and incubate for 20 min at 4 °C.
12. Wash the cell solution with FACS buffer three times.
13. Resuspend the cells in 0.5 mL of FACS buffer.
14. Add an appropriate concentration of PE-conjugated human KDR antibody and incubate for 45 min at 4 °C.
15. Wash the cell solution with FACS buffer twice.
16. Resuspend the cells in 5–10 mL of FACS buffer, and then pass through 70 μm cell strainer just before cell sorting.
17. Sort two KDR^+ subsets (KDR^+CAR^- and KDR^+CAR^+ cells) with FACS Aria or FACS AriaII (BD Bioscience).

3.3.4 Hematopoietic Differentiation from Human ESC/iPSC-Derived Cells

1. A day before sorting, prepare 24-well plates with 80–90 % confluent monolayer of OP9 cells cultured in OP9 medium.
2. Seed FACS-sorted KDR+CAR– cells on OP9 feeder layer with differentiation medium (*see* Section 2.3.6, **Step 10**) at a density of 2.0–5.0 × 10^4 cells/well.
3. After 4–7 days in culture, confirm the hematopoietic differentiation of FACS sorted KDR^+CAR^- cells by FACS and RT-PCR analyses (*see* **Note 5**).

3.3.5 Cardiac Differentiation from Human ESC/iPSC-Derived Cells

1. Prepare gelatin-coated 96-well plates, wash with PBS three times, and then dry the plates.
2. Seed FACS-sorted KDR+CAR+ cells on the gelatin-coated 96-well plates with differentiation medium-1 (*see* Section 2.3.7, **Step** 7) at a density of 0.5–1.0 × 10^5 cells/well.
3. After 2 days in culture, change the culture medium to differentiation medium-2 (*see* Section 2.3.7, **Step 8**). Do the same step every 2 days.
4. On day 7, confirm cardiac differentiation of FACS-sorted KDR^+CAR^+ cells by immunocytochemical and RT-PCR analyses (*see* **Note 6**).

4 Notes

1. Because the percentage of $Flk1^+$ cells in EBs reached peak at day 7, and decreased thereafter, 7 day-cultured EBs were used for cell sorting.
2. To confirm hematopoietic differentiation of mouse ESCs/iPSCs, we evaluate the percentage of Ter119- or

CD45-positive cells by FACS analysis, and examine the expression of marker genes, such as *Scl*, *Runx-1*, *Gata-1*, *Gata-2*, and *Fli-1* with quantitative RT-PCR analysis (*see* Tables 1–3).

3. The cardiac differentiation of mouse ESCs/iPSCs are confirmed by both the expression of a cardiac marker cTNT with immunocytochemical analysis and the expression of marker genes, such as *Tbx-5*, *Mesp-1*, *Gata-4*, *Myl-4*, *cTNT*, *aMHC* with quantitative RT-PCR analysis (*see* Tables 1–3).
4. At day 7 in culture, quantitative RT-PCR confirmed increased expression of marker genes for hematopoietic and cardiac cells. We used *Runx-1* and *Scl* as hematopoietic cell markers, and used *Isl-1* and *Tbx-5* as cardiac cell markers (*see* Tables 3).
5. We detect the hematopoietic differentiation of human ESCs/iPSCs by FACS analysis using CD45 and by quantitative RT-PCR analysis of marker genes, such as *Scl*, *Runx1*, and *Gata-2* (*see* Tables 1–3).
6. Human ESC/iPSC-derived cardiomyocytes are examined by either immunocytochemistry for cTNT and quantitative RT-PCR of marker genes *Tbx-5, Isl-1*, and *cTNT* (*see* Tables 1–3).

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Methods for Derivation of Multipotent Neural Crest Cells Derived from Human Pluripotent Stem Cells

John Avery and Stephen Dalton

Abstract

Multipotent, neural crest cells (NCCs) produce a wide range of cell types during embryonic development. This includes melanocytes, peripheral neurons, smooth muscle cells, osteocytes, chondrocytes, and adipocytes. The protocol described here allows for highly efficient differentiation of human pluripotent stem cells to a neural crest fate within 15 days. This is accomplished under feeder-free conditions, using chemically defined medium supplemented with two small molecule inhibitors that block glycogen synthase kinase 3 (GSK3) and bone morphogenic protein (BMP) signaling. This technology is well suited as a platform to understand in greater detail the pathogenesis of human disease associated with impaired neural crest development/migration.

Keywords: Neural crest cells, Human pluripotent stem cells, Human embryonic stem cells, Human induced pluripotent stem cells

1 Introduction

Neural crest cells (NCCs) arise from the neural plate border during closure of the burgeoning neural tube. NCCs delaminate from the roof plate and migrate to distinct targets throughout the developing embryo where they differentiate to form varied functional tissue (1–4). Migrating NCCs maintain a characteristic phenotype, however, NCC point of origin and local microenvironments encountered during migration influence cell and ultimately tissue fate (5–8). NCCs originate from four separate compartments (cranial, cardiac, vagal, and trunk), which lie along the rostro-caudal axis of the developing neural tube (7). As a result of the multipotent nature of NCCs, aberrations that are associated with the development of NCCs from the neural plate border, their migration within the embryo and/or their terminal differentiation lead to a wide variety of diseases or syndromes, known as neurocristopathies (9). The study of neural crest biology has clear basic science and clinical relevance: NCCs have been dubbed the “fourth germ layer” due to their multipotency. Moreover, the diseases that are a consequence of improper neural crest development or migration span many organ systems and can often have debilitating or lethal outcomes.

The strategy developed by our laboratory was influenced by previous studies focusing on neuroectoderm specification (3, 10). These approaches lead to low efficiency derivation of NCCs by utilizing concurrent inhibition of transforming growth factor (TGF)-β- and bone morphogenetic protein (BMP)-dependent signaling. The predominant cell type within these culture systems displays a neural progenitor cell (NPC) phenotype with high expression of PAX6. Moreover, these systems were laborious, often relying on feeder layer co-culture and subsequent FACS enrichment steps (3, 10–14). In an effort to alleviate these limitations, we developed a highly efficient, single-step method for the generation of NCCs from human pluripotent stem cells (15, 16). The protocol described here is performed under feeder-free conditions, requires no enrichment or selection steps and generates cultures composed of ≥90 % cells with neural crest identity. This is accomplished by inhibition of Smad1,5,8 and activation of the canonical Wnt pathway using small-molecule inhibitors of TGF-β signaling (SB431542) and glycogen synthase kinase 3 [GSK3 inhibitor IX (BIO)], respectively. In addition to highly efficient differentiation and ease of culture, the differentiated NCCs are capable of self-renewal for greater than 30 passages. NCCs are then capable of further differentiation into a multitude of additional cell types such as mesenchymal stem cells, peripheral neurons, adipocytes, smooth muscle cells, chondrocytes, and osteocytes (15, 16). Importantly, these cells are also capable of clonal expansion and can be cryopreserved with no loss in potency or self-renewing potential.

2 Materials

2.1 Medium Preparation

Special care should be made to prepare all medium using freshly thawed factors from frozen aliquots. Bulk medium preparation (greater than 500 mL) should be avoided. Unless otherwise stated, all medium should be utilized within 7 days of preparation. For the most consistent and reliable results, maintenance and differentiation medium should be prepared, as needed, every 1–2 days. When not in use, medium must be refrigerated at +4 °C.

1. Collagenase IV Dissociation Solution:
   Combine enough collagenase IV powder and DMEM/F12 (1:1) to reach a final concentration of 400 units/mL (*see* **Note 1**). Filter-sterilize the solution and store at 4 °C for a week. Alternatively, the solution may be placed in aliquots and stored at −20 °C for up to 2 months.
2. StemPro® hESC Culture Medium: StemPro® should be mixed according to the manufacturer's instructions.

3. Basal Defined Medium: For each 500 mL of basal defined medium, combine 50 mL of a 20 % (vol/vol) stock solution of Probumin®, 5 mL of penicillin (10,000 IU) and streptomycin (10,000 μg/ml) 100×, 5 mL of L-alanyl-L-glutamine (43.44 mg/mL) 100×, 5 mL of MEM non-essential amino acids, 0.5 mL of trace elements A 1,000×, 0.5 mL of trace elements B 1,000×, 0.5 mL of trace elements C 1,000× (Cellgro), 0.9 mL of 2-mercaptoethanol, transferrin (10 μg/mL), (+)-sodium L-ascorbate (50 μg/mL), and reach final volume of 500 mL by adding 432 mL of DMEM/F12 (1:1). Filter-sterilize (0.22 μm pore) and store at 4 °C for up to 2 weeks for use with maintenance and differentiation media preparation (*see* **Note 2**).
4. Human Embryonic Stem Cell (hESC) Maintenance Medium. Add the following factors to the basal defined medium with the specified final concentrations: Heregulin β-1 (10 ng/mL), Activin A (10 ng/mL), LONG® R3 IGF-I (200 ng/mL), and Fgf2 (8 ng/mL) (*see* **Notes 2** and **3**).
5. Neural crest differentiation medium. Add the following factors and inhibitors to the basal defined medium with the specified final concentrations: Heregulin β-1 (10 ng/mL), LONG® R3 IGF-I (200 ng/mL), and FGF2 (8 ng/mL), GSK3 inhibitor IX (BIO) (2 μM), and SB431542 (20 μM) (*see* **Notes 2–4**).
6. Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix -coated plates: Prepare 1 mL aliquots of Geltrex™ according to manufacturer's protocol (*see* **Note 5**). Thaw a 1 mL aliquot of Geltrex ™ and dilute it to 1:30 or 1:200 (*see* **Notes 6** and **7**) in DMEM/F12 before plating. Add 1.5 mL of the Geltrex™ dilution to a 35-mm tissue culture plate or 3 mL of the final dilution to a 60-mm tissue culture plate. Incubate coated plates at 37 °C for at least 1 h prior to use.

3 Methods

This neural crest differentiation protocol works equally well for hESCs or hIPSCs; however, cultures must be adapted to feeder-free and single-cell growth conditions.

3.1 Adaptation of hESC and hiPSC Cultures to Feeder-Free Conditions

Skip to Section 3.2 (Adaptation of hESC/hiPSC cultures to single-cell growth conditions) if hESC/hiPSC cultures are already maintained in feeder-free conditions.

It is advisable to only passage one well/plate at a time and to work as quickly as possible through each step.

1. Once colonies reach proper confluence, aspirate the culture medium and wash with 1× PBS (*see* **Note 8**); aspirate the PBS and add enough collagenase IV dissociation solution to fully cover one well/plate (*see* **Notes 9–11**).

2. Manual passage of the colonies yields the most uniform results and leads to improved survival upon passage. Many methods can be used for manual passage; however, the most facile method utilizes the StemPro® EZ™ Passage Tool (*see* **Note 12**).
3. Upon addition of the collagenase IV solution, immediately begin manual passage. Once colonies have been cut into pieces, incubate them in the collagenase solution at 37 °C for 5–30 min or until the colony fragments begin to lift up from the plate; observe the colonies under the microscope periodically to detect this change (*see* **Note 13**).
4. Gently collect the colonies using a 5 mL pipette and place in a 15 mL conical tube. If the colonies are difficult to remove by gentle pipetting, you may use a cell scraper to gently detach them from the plate (*see* **Note 14**). Once the cells have been collected in the conical tube, wash the plate with 2–5 mL of 1× PBS (depending on well/plate size) to collect remaining colonies in the dish and to dilute the collagenase in the conical tube (*see* **Note 15**). Centrifuge the cells for 4 min at 200 × *g* at RT.
5. Carefully aspirate the collagenase/PBS supernatant and resuspend the cell pellet in 4 mL of pre-equilibrated StemPro™ medium.
6. Plate the cells using a 1:4 (vol/vol) ratio (*see* **Note 16**) onto freshly prepared Geltrex-coated plates. Add pre-equilibrated StemPro® medium for a total volume of 2 mL for a 35-mm dish and a total volume of 4 mL for a 60 mm dish.
7. Maintain the cells at 37 °C in a 5 % CO_2 incubator and replenish spent medium with fresh pre-equilibrated StemPro® medium every day. Repeat steps 1–7 as necessary until colonies begin to need passaging every 4–5 days (*see* **Notes 17** and **18**). The colonies may be kept in this manner indefinitely; however, for neural crest differentiation, they must be adapted to Accutase passage for single-cell culture as described below.

3.2 Accutase® and Single-Cell Culture Adaptation

1. When hESC/hiPSC colonies have become adapted to Geltrex™, they must be further adapted to Accutase® dissociation and single-cell culture before they may be differentiated to neural crest stem cells. Aspirate the StemPro® medium and add enough Accutase® cell dissociation reagent to cover the plate (~2 mL/60 mm dish). Leave the dish at room temperature (RT) for 5–10 min, checking frequently to ensure that the colonies begin to conform to a rounded shape and the colony edges begin to detach from the plate but are still loosely adherent (*see* **Note 19**).
2. Gently collect the colonies using a 5 mL pipette and place in a 15-mL conical tube and centrifuge the cells for 4 min at 200 × *g* at RT.

3. Carefully aspirate the Accutase® supernatant and resuspend the cells in 4–5 mL of StemPro® medium, if the cells are still quite sensitive to Accutase® treatment or hESC maintenance medium, if they have become well tolerant of Accustase®. While adapting the cells to Accutase®, it is best to pass at a ratio of 1:2, until passaging is required every 4–5 days (*see* **Notes 20–22**).
4. Count the cells with a hemocytometer and replate them at a density of ~5–8 × 10^4 cells/cm^2. Once the cells have become well adapted to Accutase® passage they may be passed without cell number determination at a 1:4 to 1:5 (vol/vol) ratio on a Geltrex™-coated plate.
5. Maintain the cells at 37 °C in a 5 % CO_2 incubator and replenish spent medium with fresh pre-equilibrated medium every day. Human ESCs maintained in these conditions should remain positive for pluripotent markers such as SOX2, OCT4 and NANOG.

3.3 Differentiation of hESC to Neural Crest Stem Cells (NCCs)

1. When hESC plates have reach 75–85 % confluence, they are ready to be passaged and replated for differentiation into NCCs. Aspirate hESC maintenance medium from culture dish and add enough Accutase® cell dissociation reagent to cover the plate (~2 mL/60 mm dish). Leave the dish at room temperature (RT) for 5–10 min, checking frequently to ensure that the cells conform to a rounded shape, but are still loosely adherent (*see* **Note 19**).
2. Collect the cells in a 15 mL tube and centrifuge for 4 min at 200 × *g* at room temperature.
3. Aspirate the supernatant and resuspend the cells in 4–5 mL of pre-equilibrated hESC maintenance medium.
4. Count the cells with a hemocytometer and replate them at a seeding density of ~9 × 10^4 cells/cm^2 onto Geltrex™-coated plates in hESC pre-equilibrated maintenance medium.
5. After 24 h, aspirate the hESC maintenance medium, wash the cells with 1× PBS (*see* **Note 23**), and replace with neural crest differentiation medium.
6. Replenish spent medium with fresh neural crest differentiation medium every day.
7. Differentiating cells will reach 75–85 % confluence within 3–4 days and density/morphology should be monitored daily. Morphological changes should become apparent around days 4–5 (*see* Fig. 1a) after exposure to neural crest differentiation medium, and subsequent neural crest morphology should become apparent between 7 and 12 days of differentiation in neural crest differentiation medium (*see* Fig. 1a).

8. Upon reaching proper confluence (75–85 %), typically every 3–4 days, the differentiating cells should be passed using Accutase® according to the method described above and continued to be reseeded in neural crest differentiation medium at the same density.

9. NCC identity can be analyzed as early as 15 days post initial exposure to neural crest differentiation medium However, it may take up to 21 days to reach full maturity (*See* Fig. 1). Analyses include immunocytochemistry, flow cytometry and/or RT-PCR (Fig. 1b–d). If you are using immunocytochemistry, NCCs should be positive for markers such as p75, Hnk1, AP2. Flow cytometric analysis of NCCs should yield p75$^+$ and HNK1$^+$ cell populations. If you carry out RT-PCR, NCCs should express genes such as PAX3, AP2, ZIC1, SOX9, and SOX10, among others (*See* Fig. 1).

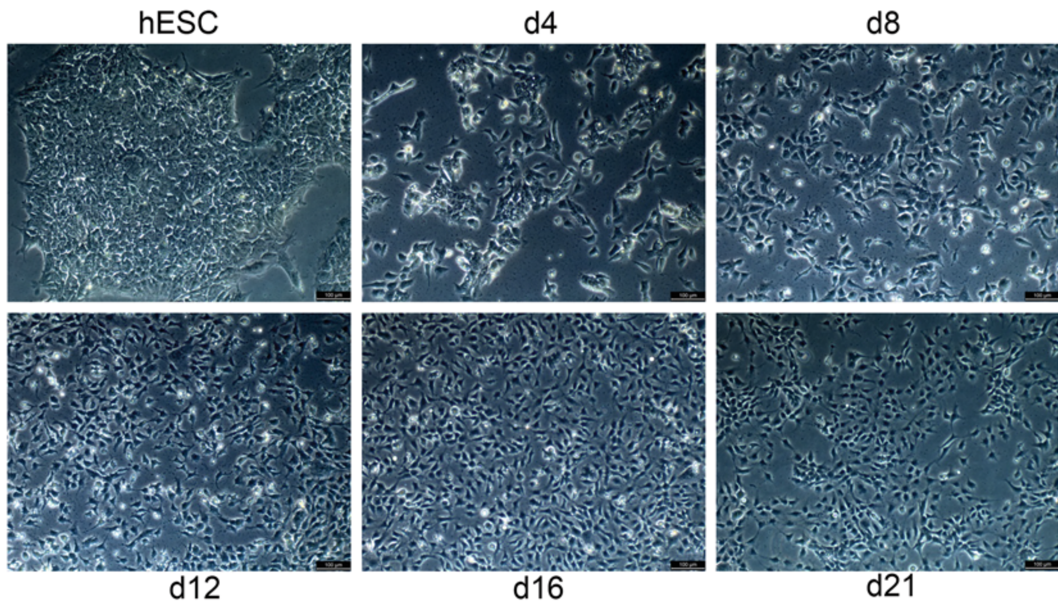

Fig. 1 Characteristics of NCSC differentiation from hPSCs. Morphological appearance of NCSCs during differentiation: NCSCs lose tight colony morphology, cell size becomes reduced, cell shape becomes increasingly stellate, and the cellular nucleus–cytoplasm ratio is reduced relative to hPSCs, 10× magnification, scale bar = 100 μm (**a**); Transcript markers during NCSC during differentiation. Levels are shown relative to that in undifferentiated hESCs (WA09). The *x*-axis corresponds to day of differentiation. NCSCs transcripts increase as early as d3 (SOX9) with maxima reached as the differentiation proceeds from d15-d18. Once the NCSC phenotype has been reached, expression patterns stabilize (**b**); Immunofluorescence of NCSC cell surface markers (HNK1 and p75) and transcription factor (AP2) at d18, 20× magnification, scale bar = 50 μm (**c**); Flow cytometric analysis of NCSC cell surface markers during differentiation (**d**)

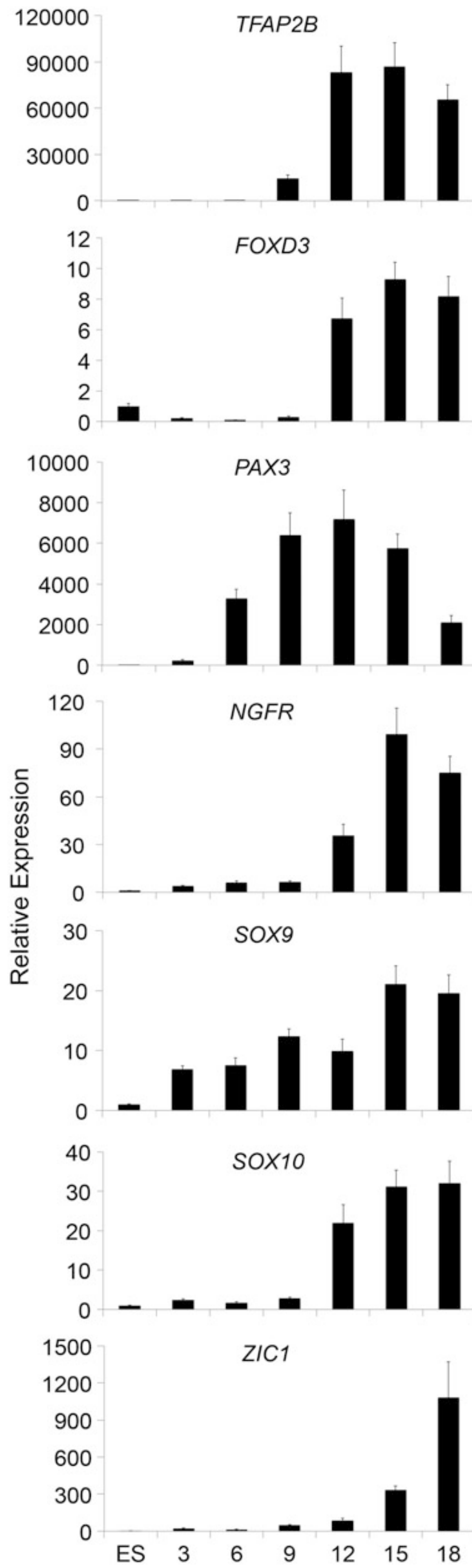

Fig. 1 (continued)

c

DAPI MERGE

HNK1

P75

AP2

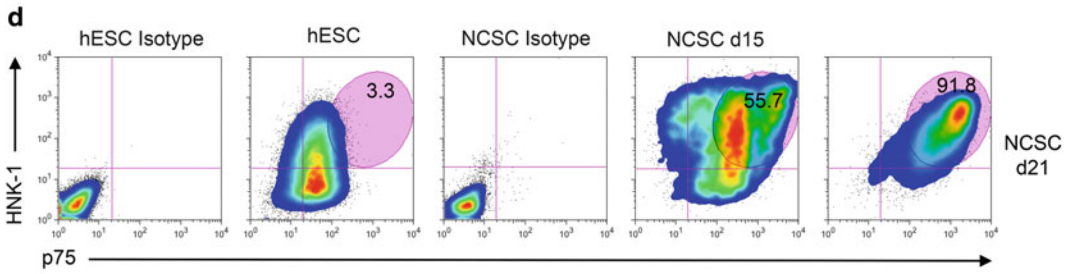

Fig. 1 (continued)

4 Notes

1. If unit concentrations of collagenase IV are not given, use 1 mg/mL.
2. To ensure proper concentration of growth factors, it is best to follow strict aseptic technique with no need to filter the medium; however, if factors or other reagents are shared or their handling/aliquoting cannot be accounted for, the medium must be filter-sterilized using a 0.22 μm pore.
3. Media should be pre-equilibrated to 37 °C prior to use.
4. The use of commercially available stem cell media, such as StemPro® or mTesR®, is not recommended for this protocol, as the presence of Activin A and/or TGF-β inhibits efficient NCC differentiation. Additionally, the use of serum-rich or KSR media is also not recommended due to the undefined nature of their components and poor efficiency in NCC yield.
5. In our lab, we initially aliquot 1 mL containing a 1:1 solution of Geltrex™–DMEM/F12 by adding 5 mL of ice-cold DMEM/F12 to 5 mL of frozen Geltrex™ and allow the mix to completely thaw on ice before thoroughly mixing by pipetting. It is important to work quickly as Geltrex™ will gel in 5–10 min at temperatures above 15 °C. To avoid the solution reaching this temperature, we keep the aliquoted tubes on ice until we finish portioning out the solution. These aliquots are immediately frozen (−20 °C) for later use.
6. When adapting cells to feeder free conditions, we utilize a 1:30 dilution of Geltrex™ to DMEM/F12. This is met by diluting a 1 mL aliquot of 1:1 Geltrex™–DMEM/F12 as in **Note 5** into a further 14 mL of DMEM/F12 for a final volume of 15 mL. The cellular stress upon change from the feeder layer to Geltrex™ appears to be lessened by using this higher concentration, as cell survival is enhanced. After two to three passages, the cells may be transitioned further to a Geltrex™–DMEM/F12 dilution of 1:200. Cell survival and spontaneous differentiation are unaffected, while considerable cost savings can be attained by this increased dilution.
7. For best results, coated plates may be kept for five days at 37 °C in a 5 % CO_2 incubator, provided the plates are not allowed to dry out. Take care to monitor coated plates and add additional DMEM/F12 if needed after solidification to prevent drying. Alternatively, the plates may be wrapped with Parafilm™ and stored at 4 °C for no greater than 2 weeks.
8. You may use PBS with or without Ca^{2+}/Mg^{2+}, as they do not affect collagenase activity. The wash step is included to rid the plate of components that may inhibit or reduce collagenase IV activity, such as Fe^{2+}.

9. Prior to beginning any plating or dissociation work with cells, ensure that you have Geltrex-coated plates prepared and that they are ready to be utilized.
10. Prior to adding the dissociation solution it is critical to evaluate the colonies for spontaneous differentiation. Observe the plate under an inverted scope and identify colonies that show evidence of differentiation or are too large (excessively large colonies are more prone to differentiation). Use an indelible marker to indicate on the plate where the colonies you wish to remove are so that you may remove them in the biological safety cabinet. Appropriate colony morphology is indicated by round, well-defined edged, homogenous colonies that are tightly packed; the cells within retain a characteristic high nuclear to cytoplasmic ratio and display easily identifiable nucleoli. Remove unwanted colonies by first aspirating the culture medium then etching a line around and through the colony(ies) using a 20 μL pipette tip or 20–22 G-1½ needle (this guards against removing the underlying feeder layer in a sheet and prevents removing the colonies you wish to pass). Using a sterile Pasteur pipette, gently slide the pipette along the colony edges and center, being careful to observe and prevent any pulling from the feeder layer. It is critical to work quickly to avoid drying of the plate. Once the colonies are removed, wash with 1× PBS; replace aspirated PBS with fresh hESC maintenance medium. If more than 10 % of the colonies in a well/plate are of an inappropriate morphology, the culture is unreliable and should be discarded; you must examine your media preparation as well as your technique to maintain proper hESC identity.
11. When working with multiple wells/plates of cells, it is critical to add the cell dissociation solution one well/plate at a time. For example, if using a 6 well plate, add the solution to one well and proceed to the next step with that well before moving on to the next well. This is time consuming but yields the most reliable results and protects cells from over exposure to the dissociation solution.
12. The StemPro® EZ™ Passage Tool separates the colonies into optimally sized, uniform pieces, which survive passage with greater frequency and yield "goldilocks" colonies that are not too large or too small-leading to decreased spontaneous differentiation and more reliable culture. When attempting to pass several wells/plates the StemPro® EZ™ Passage Tool allows the greatest time-savings and therefore the least amount of exposure of the culture to contaminants. Use one Tool per well/plate.

13. Once you have scraped the colonies in one well containing the collagenase solution, you may begin the process for other wells/plates now—do *not* exceed 6 wells/plates at one time, as time will become a limiting factor for good technique and ultimately culture survival/reliability.
14. Excessive, vigorous pipetting will damage colonies or reduce their size to suboptimal dimensions. Gentle pipetting 2–3× should be enough to remove the colonies from the plate. If this is not enough, you may not have incubated the cells in collagenase solution long enough. You may use gentle scraping with a sterile cell scraper, but make sure to add PBS or hESC medium to the plate first.
15. Adding PBS to the collagenase solution accomplishes two goals: (1) it makes aspirating the supernatant from the conical tube after centrifugation easier, as an undiluted viscous collagenase solution can easily pull the entire pellet with it, and (2) it further dilutes the collagenase to be removed—if collagenase is not removed, the colonies will not adhere well, thus leading to reduced plating efficiency and spontaneous differentiation.
16. It is advisable to use two Geltrex™ plates and two MEF plates and place 1 mL of the 1:4 (vol/vol) colony/StemPro® dilution onto each plate; that way, the two MEFs plates serve as reserves in the event the cells do not survive well on the new Geltrex™ plates. If the colonies are successful upon transitioning to Geltrex™, then the two reserve MEF plates make great frozen stocks should you need to return to MEF plates in the future.
17. Reaching a point at which colonies need to be passed every 4–5 days may take 2–3 weeks for reliable passaging and survival on Geltrex™.
18. As with culturing hESC colonies on feeders, passage the colonies when any of the following criteria are observed: (1) colonies are becoming too large, (2) colonies are becoming too dense (proximity to neighboring colonies decreases or colonies begin touching), (3) spontaneous differentiation begins, or (4) 10 days pass between passages and the colonies have not violated the three preceding criteria.
19. Overly confluent cells require longer Accutase® incubation; it is not optimal for the cells to fully detach, and not to pipette too much, as this decreases cell health [viability??].
20. The cells may take up to 3 weeks to become adapted to Accutase treatment.
21. In order to subsequently differentiate the cells, they should be passaged in hESC maintenance medium (not in a commercial stem cell medium) at least twice prior to switching to neural crest differentiation medium. Differentiation efficiency is

dramatically reduced if the cells have not been adapted to the maintenance medium prior to any attempts at differentiation toward NCCs.

22. Avoid exceeding densities over 85–90 % confluence in single cell culture conditions, as this dramatically increases spontaneous differentiation and decreases reliable differentiation to lineage specific cell types later on. Also, it is best not to plate at densities which lead to excessive passage, i.e., cultures should not need passaging more than every 4 days.
23. It is critical to wash the cells with PBS in order to remove any remaining Activin A in the medium. The presence of any Activin A will reduce the NCC differentiation.

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From Naive to Primed Pluripotency: In Vitro Conversion of Mouse Embryonic Stem Cells in Epiblast Stem Cells

Matteo Tosolini and Alice Jouneau

Abstract

Mouse embryonic stem cells (ESCs) derive from the inner cell mass (ICM) of a blastocyst at E3.5 while mouse epiblast stem cells (EpiSCs) derive from the late epiblast of a post-implantation embryo at E5.5–E7.5. Both cells are able to differentiate into derivatives of the three germs layers but only ESCs are able to produce chimeras when they are introduced into a blastocyst. To support the naive state of pluripotency, ESC culture requires Leukemia inhibitory factor (Lif) and either serum or inhibitors of Erk and Gsk3 pathways (2i) while the primed pluripotency of EpiSCs is maintained using Activin A and Fibroblast Growth Factor 2 (FGF2). It is possible to obtain EpiSCs in vitro starting from ESCs but also to induce ESCs starting from EpiSCs even if this second process is very difficult and inefficient. In this protocol we describe how we perform the process of conversion from ESCs to EpiSCs.

Keywords: Mouse embryonic stem cells (ESCs), Epiblast stem cells (EpiSCs), Conversion, Collagenase, Chemically defined medium (CDM)

1 Introduction

From a mouse embryo at different stages it is possible to capture two types of pluripotent cells: mouse embryonic stem cells (ESCs) from the inner cell mass (ICM) of a blastocyst at E3.5 and epiblast stem cells (EpiSCs) from the late epiblast layer of a post-implantation embryo at E5.5–E7.5 (1, 2). Both types of cells are pluripotent as they are able to differentiate into derivatives of all three germ layers either in vitro or in vivo through teratoma, but only ESCs are able to produce chimeras when they are injected into a blastocyst (3). ESCs and EpiSCs share the expression of the core pluripotency factors: Oct4, Nanog, Sox2, but ESCs express some naive pluripotent factor like Rex1 and Klf4 which are absent in EpiSCs, while EpiSCs express some epiblast specific genes such as Fgf5 and Otx2 which are already markers of differentiation absent in ESCs. A strong epigenetic difference between naive and primed state of pluripotency is that female ESCs have two active X chromosome while female EpiSCs present already one inactive X chromosome. To be maintained in vitro, ESCs required serum and Leukemia inhibitory factor (Lif), an activator of the transcriptional factor Stat3 which inhibits

differentiation and promotes self-renewal of naive pluripotency (4). On the other hand, EpiSCs do not respond to Lif while they required Activin A and Fibroblast Growth Factor 2 (FGF2) to sustain primed pluripotency in vitro (2, 5). EpiSCs share with ESCs a large nuclear-to-cytoplasmic ratio and prominent nucleoli, but their morphology is more two-dimensional and epithelial. In addition EpiSCs do not survive efficiently as isolated single cells but they need to be passaged in clumps, all these characteristics of primed pluripotency in mouse are shared with human embryonic stem cells (hESCs) (6). A recent study of our laboratory has shown that the two states of mouse pluripotency differ also in terms of DNA methylation, in particular EpiSCs present a higher proportion of methylation at a subset of gene promoters compared to ESCs and some of these genes are specific to the naive pluripotency (7). It is possible to convert in vitro ESCs into EpiSCs only by changing the culture conditions (8). Interestingly the in vivo transition from naive to primed pluripotency takes only 2 days while in vitro the conversion process needs about a week to obtain stable colonies morphologically similar to EpiSC (Fig. 1). However, a recent study has shown that within 2 days of conversion, the cells extinguish the expression of naive pluripotent genes, while adopting transiently the identity of an early epiblast (9). Moreover it is possible in vitro to induce the opposite process performing a reversion from EpiSCs to ESCs, also by switching the culture conditions, but this process is

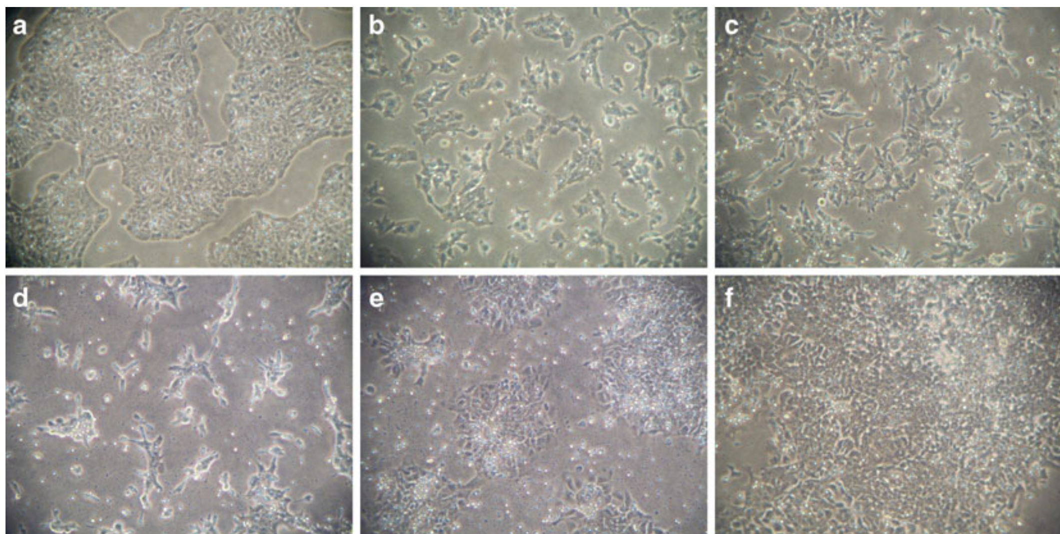

Fig. 1 Morphology of ESCs during conversion to EpiSCs under the phase contrast microscope. (**a**) ESCs in CDM/2i/LIF. (**b**) Converted ESCs (cESCs) at day 1 of conversion. (**c**) cESCs at day 2 of conversion. (**d**) cESCs at day 3 of conversion (high rate of mortality). (**e**) cESCs at day 5 of conversion: first appearance of EpiSC-like colonies before the collagenase treatment. (**f**) cESCs at day 7 of conversion (after the first passage): big EpiSCs colonies

inefficient and long, suggesting the presence of an epigenetic barrier as during the reprogramming from somatic to pluripotent cells (10). Conversion from ESC to EpiSC is a useful model to study the molecular and epigenetic events that trigger the transition from the naive to the primed pluripotency (9, 11–13). In the last few years the discovery of two small molecules that inhibit the MAPK and Gsk-3 pathways and could substitute serum in ESCs culture makes a great change in the field (14). With this new completely defined (2i) culture condition, ESCs displayed a state of pluripotency closer to that of the ICM (15, 16). One of the advantages of using a chemically defined medium (CDM) is that the same basal medium can be used to convert ESC to EpiSC, with the only change being the added factors, in this specific case from 2i/LIF to Activin A/FGF2.

2 Materials

2.1 Materials

1. Conical centrifuge tubes 15 and 50 mL, sterile.
2. Graduated plastic pipettes (sterile, single package) of 2, 5, 10, and 25 mL.
3. Glass Pasteur pipettes sterilized in an aluminum container using a dry oven (4 h at 180 °C).
4. Plastic sterile Petri dishes for cell culture of 35 and 60 mm of diameter (*see* **Note 1**).
5. Pipettes P1000, P200, P20, P10 and sterile plastic tips.
6. 20 mL syringes.
7. Syringe membrane filters, 0.22 μm, in PES (Polyethersulfone).
8. Water bath.
9. Centrifuge (for 15 mL plastic tubes).
10. Incubator at 37 °C in a humid atmosphere with 5 % CO_2.
11. Vertical laminar flow hood.
12. Aspiration system.
13. Phase-contrast microscope and Neubauer chamber or other cell counting method.
14. H_2O Milli-Q produced with a resistivity of 18.2 MΩ cm at 25 °C and sterilized.
15. Dimethyl sulfoxide (DMSO) ≥99 %.
16. Dulbecco's Phosphate-Buffered Saline (DPBS) 1× sterile without Ca^{2+} and Mg^{2+}.
17. Trypsin–EDTA (0.25 %). Aliquots of 10 mL stored at −20 °C.

18. Collagenase from *Clostridium histolyticum*. Powder stored at −20 °C.
19. Protease-free BSA (Bovine serum albumin). Powder stored at +4 °C.
20. 2-mercaptoethanol (50 mM). Aliquots of 1 mL stored at +4 °C.
21. Transferrin. Resuspended in H_2O Milli-Q at the final concentration of 30 mg/mL. Aliquots stored at −20 °C.
22. Recombinant Insulin. Resuspended in H_2O Milli-Q at the final concentration of 10 μg/mL. Aliquots stored at −20 °C.
23. Recombinant Fibroblast Growth Factor-basic (FGF2). Resuspended in DPBS with 0.1 % BSA and 1 mM DTT at the final concentration of 12 μg/mL. Aliquots of 20 μL stored at −20 °C.
24. Activin A. Resuspended in DPBS with 0.1 % BSA at the final concentration of 20 μg/mL. Aliquots of 20 μL stored at −20 °C.
25. Ham's F-12 Nutrient Mix 1×, supplemented with 2 mM of L-glutamine. Stored at +4 °C.
26. Iscove's Modified Dulbecco's Medium (IMDM) 1×, supplemented with 2 mM of L-glutamine. Stored at +4 °C.
27. Dulbecco's Modified Eagle Medium (DMEM) 1×, supplemented with 2 mM of L-glutamine. Stored at +4 °C.
28. FBS (Fetal bovine serum). Stock stored at −80 °C, while aliquots of 50 mL at −20 °C.
29. Chemically Defined (CD) Lipid Concentrate. Aliquots of 10 mL stored at +4 °C.
30. 1-Thioglycerol ≥97 %. Aliquots of 50 μL stored at +4 °C.

2.2 Collagenase II Solution

Collagenase II solution is prepared with: 50 % IMDM 1× (supplemented with 2 nM of L-glutamine), 50 % Ham's F-12 Nutrient Mix 1× (supplemented with 2 nM of L-glutamine), 3.5 mg/mL of collagenase from *Clostridium histolyticum* (*see* **Note 2**). The solution is then sterilized by filtering with 0.22 μm PES membrane filter. Collagenase II solution can be kept for 1 month at +4 °C.

2.3 Chemically Defined Medium (CDM)

CDM is prepared with: 50 % IMDM 1× (supplemented with 2 mM of L-glutamine), 50 % Ham's F-12 Nutrient Mix 1× (supplemented with 2 mM of L-glutamine), 5 mg/mL BSA (*see* **Note 3**), 1 % CD lipid concentrate, 450 μM of 1-thioglycerol, 7 μg/mL recombinant insulin, 15 μg/mL transferrin. The CDM is then sterilized by filtering with 0.22 μm PES membrane filter. CDM can be kept for 1 month at +4 °C.

2.4 Serum-Containing Medium

This medium will be used to inactivate the trypsin and as a source of extracellular matrix to coat the dishes for converting cells. It contains a basal medium such as DMEM, IMDM or F12 supplemented with 10 % FBS. It can be kept for 2 months at 4 °C.

3 Methods

All the cell culture work is performed under sterile condition: manipulation of cells and preparation of solutions are done under a vertical laminar flow hood. ESCs and EpiSCs are cultured at 37 °C in a humid atmosphere with 5 % of CO_2.

3.1 Conversion from ESCs to EpiSCs

1. Incubate 35 mm dishes with 1 ml of serum-containing medium for at least 1 h at 37 °C (*see* **Note 4**).
2. Pre-warm Trypsin–EDTA (0.25 %), serum-containing medium and CDM in the water bath at 37 °C.
3. Aspirate serum-containing medium from the dishes with a sterile glass Pasteur pipette.
4. Perform a DPBS wash (1 mL for dishes of 35 mm).
5. Replaced DPBS by CDM (1.5 mL for dishes of 35 mm) supplemented freshly with 12 μg/mL of FGF2 and 20 μg/mL of Activin A and put the dishes in the incubator for equilibration.
6. Aspirate the old medium from the dishes with ESCs (*see* **Note 5**).
7. Quickly wash cells with DPBS kept at room temperature (*see* **Note 6**).
8. Add Trypsin–EDTA (0.25 %) on ESCs and incubate at 37 °C for 2 or 3 min according to their condition, serum or 2i respectively, to detach cells from the dishes.
9. Add on top the same volume of serum-containing medium and completely dissociate the cells by pipetting several times with a P1000 pipet.
10. Take a little amount of solution with ESCs singularized to count them.
11. Transfer the cells into a 15 mL plastic tube and centrifuge 5 min at 200 g at room temperature.
12. Aspirate the supernatant and thoroughly resuspend the visible cell pellet with 1 mL of CDM by pipetting with P1000 pipette and centrifuge another time for 5 min at 200 g at room temperature.
13. Aspirate the supernatant and resuspend ESCs in the appropriate amount of CDM according to the number of cells counted.
14. Finally plate 1.5 millions of ESCs in the new dishes coated with serum or fibronectin and containing CDM supplemented with FGF2 and Activin A.

15. Every day replace the old medium with fresh CDM supplemented with the factors (*see* **Note 7**).
16. At the fifth day of conversion there is normally the appearance of first EpiSCs-like colonies (already quite flat or more ball-like ones) and they need to be passaged in order to help them to spread (Fig. 1). If the colonies are still small, wait one more day before passaging.

3.2 Passaging of EpiSCs Colonies

1. Prepare the new plates as in 3.1 (**steps 1, 3–5**).
2. Pre-warm collagenase II solution and CDM in the water bath at 37 °C.
3. Aspirate the old medium from the dishes.
4. Wash cells with DPBS kept at room temperature.
5. Add collagenase II solution on the EpiSC-like cells (400 μL for dishes of 35 mm) and incubate for 30 s at room temperature (*see* **Note 8**).
6. Wash with DPBS.
7. Flush the EpiSC-like colonies using 1 mL of CDM and a P1000 pipette in order to detach them in clumps without pipetting and singularizing cells.
8. Transfer the cells into a 15 mL plastic tube (*see* **Note 9**) and centrifuge 5 min at 200 g at room temperature.
9. Aspirate the supernatant and resuspend very gently the cells in CDM just with one or two pipetting of P1000 pipette in order to keep EpiSCs colonies in clumps and not single cells.
10. Transfer EpiSC colonies in the newly prepared dishes (*see* **Note 10**).

4 Notes

1. Test different types of plastic petri dishes for EpiSCs culture to check for optimal growth and absence of differentiation along passages.
2. To help dissolution of collagenase from *Clostridium histolyticum* leave the solution few minutes in the water bath at 37 °C before filtering it. The warm solution is naturally cloudy.
3. It is necessary to test different batches of BSA for EpiSCs culture to check for optimal growth and absence of differentiation.
4. The serum-containing medium can be replaced by fibronectin: use a dilution of 20 μg/ml in DPBS and incubate the dishes at least 20 mn at 37 °C.
5. The conversion can be performed starting from ESCs either cultured in 2i/Lif or in serum/Lif conditions. However,

conversion seems to induce less mortality when starting from ESCs cultured in 2i/Lif.

6. This step is necessary only if the ESCs are in serum condition.
7. During conversion there is a high rate of cell mortality so in this case it is better to wash with DPBS before changing the medium in order to eliminate the maximum of dead cells.
8. When the collagenase II solution is fresh-made, it is very active; so even 20 s will be sufficient to detach EpiSCs colonies from dishes.
9. Repeat 3.2 **step 7** more than once using another 1 mL of CDM in order to detach the maximum of EpiSCs colonies trying not to break them too much.
10. For the first passaging at the fifth day of conversion the suspension is not diluted as there is still a lot of mortality. For the next passages dilution will be 1/2 then 1/3 every 2–3 days approximately.

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In Vitro Differentiation of Embryonic Stem Cells into Hematopoietic Lineage: Towards Erythroid Progenitor's Production

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Abstract

Embryonic stem cells (ESCs) differentiation via embryoid body (EB) formation is an established method that generates the three germ layers. However, EB differentiation poses several problems including formation of heterogeneous cell populations. Herein, we described a differentiation protocol on enhancing mesoderm derivation from murine ESCs (mESCs) using conditioned medium (CM) from HepG2 cells. We used this technique to direct hematopoiesis by generating "embryoid-like" colonies (ELCs) from murine (m) ESCs without following standard formation of EBs. Our CM-mESCs group yielded an almost fivefold increase in ELC formation ($p \leq 0.05$) and higher expression of mesoderm genes;-Brachyury-T, Goosecoid, and Flk-1 compared with control mESCs group. Hematopoietic colony formation from CM-mESCs was also enhanced by twofold at days 7 and 14 with earlier colony commitment compared to control mESCs ($p \leq 0.05$). This early clonogenic capacity was confirmed morphologically by the presence of nucleated erythrocytes and macrophages as early as day 7 in culture using standard 14-day colony-forming assay. Early expression of hematopoietic primitive (ζ-globin) and definitive (β-globin) erythroid genes and proteins was also observed by day 7 in the CM-treated culture. These data indicate that hematopoietic cells more quickly differentiate from CM-treated, compared with those using standard EB approaches, and provide an efficient bioprocess platform for erythroid-specific differentiation of ESCs.

Keywords: Embryonic stem cells, Cell culture techniques, Cell differentiation, Hematopoiesis, Erythropoiesis

1 Introduction

Embryonic stem cells (ESCs) spontaneously differentiate in vitro through formation of embryoid bodies (EBs), tissue-like spheroids cultivated in suspension culture (1–3). EB formation recapitulates some aspects of early embryogenesis, including the formation of a complex three-dimensional arrangement wherein cell–cell and cell–matrix interactions support the development of the three embryonic germ layers—mesoderm, ectoderm, and endoderm. Following EB formation, cells are returned to adherent culture conditions in order for directed differentiation to occur (4–6). However, EB-based methodologies are imprecise since spontaneous differentiation into the three germ layers is not controlled (4, 5). Blood is derived from

mesoderm in direct competition with spontaneous angiogenic and cardiomyogenic differentiation (6–9). To recapitulate the process of blood formation from mesoderm in vitro, ESCs are allowed to form EBs for *5–8 days followed by terminal hematopoietic differentiation through the use of various supplements and cytokines (3, 6, 10). Recent efforts in in vitro red cell production starting from ESCs with a view to clinical applications have resulted in successful serum-free cultures for hematopoietic differentiation and the production of functional nucleated red blood cells (11–13). However, these methods have all used EB formation and coculture with feeder cells or conditioned media (CM). Non-EB cultures of hematopoietic cells derived from ESCs can also be achieved with coculture using stromal cells (11, 14–16). Although coculture provides microenvironmental cues for differentiation of ESCs, the method is labor-intense and problematic in the separation of feeders from differentiated cells and the possible formation of heterokaryons within the culture (17). Recombinant cytokines are used for more defined culture conditions for the controlled differentiation of mesoderm from ESCs. However, the method is costly and can, paradoxically, lead to multilineage development due to the redundant action of cytokines and morphogens, such as BMP-4 and Activin-A (5, 18). Alternatively, CM from the culture of differentiated somatic cells contain soluble factors, although largely undefined, that direct ESC differentiation into layers such as mesoderm in vitro and are less resource-intensive, making it a practical alternative for the study of mesoderm derivatives in vitro (5, 17, 19–21). We have previously demonstrated that CM, obtained from cultures of the human hepatocarcinoma cell line HepG2, enhanced mesoderm formation from murine ESCs (mESCs) without EB formation (5). HepG2 cells have characteristics similar to those of visceral endoderm, an early organizer during embryogenesis that secret signaling molecules specifying cell fate (19, 22, 23). The similarities between liver carcinoma cell lines and visceral endoderm have led to the suggestion that HepG2 CM reiterate visceral endoderm-like signaling in this system (5, 19). Differentiation of mESCs cultured in HepG2 CM resulted in a restricted repertoire of cell types comprised of mesoderm and parietal

*Keller et al. 1993 has reported that ESCs efficiently undergo differentiation in vitro to mesoderm and hematopoietic cells that this in vitro system recapitulates day 6.5 to 7.5 of mouse hematopoietic development. Embryonic stem cells differentiated as embryoid bodies (EBs) develop erythroid precursors by day 4 of differentiations, and by day 6, more than 85% of EBs contain such cells. The number of the EPO responsive precursors increased by day 8 of differentiation, them remained constant, and finally began to decline by day by day 12. Therefore, we hypothesized that day 5-8 are the period of mesoderm differentation in EB to recapitulate embryonic development based on the previous study reported.

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endoderm providing an efficient method for enriched mesoderm formation in vitro (5, 24). Herein, we extend our studies of directed mesoderm differentiation from mESCs using HepG2 CM and present an efficient method for the in vitro development of the hematopoietic lineage, as an alternative process for potential study and application of in vitro erythropoiesis from ESCs.

2 Materials

2.1 Tissue Culture

1. Undifferentiated E14 Tg2α murine ES and Hepatocarcinoma (HepG2) cell lines (ATCC).
2. Culture flask with tissue culture-treated low-toxin (endotoxin <0.5 endotoxin units per milliliter [EU/mL]) high-grade polystyrene culture surfaces (Nunc; T-75 flasks)
3. 35 × 10 mm culture petri dishes (Corning nontreated petri dishes [available through Sigma-Aldrich, UK]).
4. Blunt-end needles (Stem Cell Technologies, UK).
5. Leukemia inhibitory factor (LIF; ESGRO) (Chemicon [available through Milipore, UK]).
6. Gelatine solution type B from bovine skin 2 % (Sigma-Aldrich, UK). Diluted the concentrated solution with tissue culture-grade phosphate-buffered saline (PBS) to 0.1 %. For example, increase volume of 1 mL concentrated solution to 20 mL by tissue culture-grade PBS.
7. Dimethyl sulfoxide Hybri-Max (DMSO) (Sigma Aldrich, UK).
8. 1× tissue culture-grade PBS without calcium or magnesium (Gibco Invitrogen Life Technologies, UK).
9. High-glucose Dulbecco's modified Eagle's medium (DMEM) (1×) contains 4,500 mg/L glucose, pyridoxine hydrochloride and L-glutamine, but no sodium pyruvate (Gibco Invitrogen Life Technologies, UK).
10. Heat-inactivated fetal bovine serum (FBS) characterized and screened for ESCs growth (batch tested) (Gibco Invitrogen Life Technologies, UK).
11. 200 mM L-glutamine (Gibco Invitrogen Life Technologies, UK).
12. Tripsin-ethylenediaminetetraacetic acid (EDTA) solution: 0.05 and 0.25 % trypsin/0.53 mM EDTA in 0.1 M PBS without calcium or magnesium (Gibco Invitorgen Life Technologies, UK).
13. 100 units/mL penicillin and 100 μg/mL streptomycin (Gibco Invitrogen Life Technologies, UK).

14. Water for embryo transfer, sterile-filtered, BioXtra (Sigma-Aldrich, UK).
15. 14.3 M mercaptoethanol 2-(ME) (Sigma-Aldrich); dilute in the solution into 100 mM by adding 40 mL concentrated solution into 4,960 mL water for embryo transfer.
16. Iscove's Modified Dulbecco's Medium (IMDM) with high glucose and sodium pyruvate (Gibco Invitrogen Life Technologies, UK).
17. 0.1 mM Nonessential Amino Acids (Sigma-Aldrich, UK).
18. 100 mM monothioglycerol (MTG) (Sigma-Aldrich, UK).
19. 1% BIT (1 % BSA, 10 mg/mL Insulin, and 200 mg/mL Transferrin; Stem Cell Technologies, UK).
20. Growth factors: mouse stem cell factor (mSCF), mouse interleukin (mIL)-3, human (h) IL-6, and human erythropoietin (hEPO; all supplied by R&D Systems, UK).
21. Es-Cult M3120 medium (Stem Cell Technologies, UK).
22. 15- and 50-mL polypropylene conical centrifuge tubes (VWR International, UK).
23. Cryogenic vials (VWR International, UK).
24. Ethanol 96–99 % (absolute) GPR (VWR International, UK).
25. Virkon (VWR International, UK).
26. 100 % isopropyl alcohol bath (Nalgene Mr. Frosty, UK).

2.1.1 Media

1. *mESCs maintenance medium* : High-glucose DMEM supplemented with 10 % v/v FBS, 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin, 0.1 mM 2-ME and 0.1 mM LIF immediately before use. For example, add 50 mL FBS, 5 mL L-glutamine, 5 mL penicillin and streptomycin to 440 mL high-glucose DMEM. Add 1 μL 100 mM 2-ME per milliliter and 1 μL 1,000 U LIF per milliliter of the culture medium immediately before use (*see* **Note 1**).
2. *HepG2 maintenance medium*: High glucose DMEM without sodium pyruvate supplemented with 10 % FBS, 2 mM L-glutamine and 100 units/mL penicillin and 100 μg/mL streptomycin.
3. Primary differentiation medium: High glucose IMDM with sodium pyruvate supplemented with 1 % Es-Cult, 15 % FBS, 2 mM L-glutamine, 150 μM MTG, and 40 ng/mL mSCF.
4. *Feeding medium*: Primary differentiation medium supplemented with 160 ng/mL mSCF, 30 ng/mL mIL-3, 20 ng/mL h IL-6, and 3 U/mL hEPO.
5. *Hematopoietic differentiation medium*: High glucose IMDM with sodium pyruvate supplemented with 15 % FBS, 2 mM L-Glutamine, 150 mM MTG, 1 % BIT, 150 ng/mL mSCF,

30 ng/mL mIL-3, 30 ng/mL hIL-6, 3 U/mL hEPO, and 1 % Es-Cult medium.

2.1.2 General Cell Maintenance Procedure

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

1. Maintain the cells in an incubator at 37 °C in a humidified atmosphere of 5 % CO_2/95 % air.
2. Control the water level in the incubator's tank daily.
3. Check cells daily under inverted microscope. If any contamination is observed in the cultured cells, then take the culture plate out the incubator and apply 10 % virkon for approximately 1–2 h and then discard the plate.
4. After using a tissue culture hood, wipe it with 1 % virkon and aspirate some 10 % virkon for decontamination of tube and contents of canister.
5. Discard content of canister after 1–2 h treatment with virkon.

2.2 Reverse Transcriptase Polymerase Chain Reaction

1. Reverse transcriptase polymerase chain reaction (RT-PCR)-grade water (Ambion, UK).
2. Total RNA Isolation kit, RNeasy mini kit (Qiagen, UK). This kit contains the following ready-made buffers and columns:
   (a) Lysis buffer: Buffer RLT, add 10 μL of β-ME for working solution.
   (b) Buffer RW1.
   (c) Washing buffer: Buffer RPE, add 4 volumes of absolute ethanol (96–99 %) for working solution.
   (d) RNase-free water.
   (e) Qiashredder spin column.
   (f) RNeasy spin column.
3. Tissue culture-grade 1× PBS without calcium or magnesium (Gibco Invitrogen Life Technologies, UK).
4. RNase-, DNA-, adenosine triphosphate (ATP)-, and pyrogen-free 1.5 mL Eppendorf tube (VWR International, UK).
5. Cuvette (Eppendorf, UK).
6. Spectrophotometer (Eppendorf Biophotometer, Germany).
7. TE Buffer at pH 7.0 (Ambion, UK).
8. Molecular biology-grade ethanol (VWR International, UK).
9. Reverse Transcription (RT) system kit (Promega, UK). This kit contains the following reagents:
   (a) $MgCl_2$, 25 mM.
   (b) Reverse Transcription 10× Buffer.

   (c) dNTP Mixture, 10 mM.
   (d) Recombinant RNasin® Ribonuclease Inhibitor.
   (e) AMV Reverse Transcriptase (High Concentration).
   (f) Random Primers.
   (g) Nuclease-Free Water.

10. Mastermix PCR reagents contains (all by Invitrogen, UK):
    (a) 1× PCR Buffer.
    (b) 0.2 mM dNTP mixture.
    (c) 1.5 mM $MgCl_2$.
    (d) 0.2 μM each primer (forward and reverse).
    (e) 1 unit of Platinum Taq DNA Polymerase.
    (f) DNase free water.
11. Agarose powder (Invitrogen, UK).
12. 10 μg/mL Ethidium bromide (Sigma-Aldrich, UK).
13. Gel-documentation system with ultra-violet light (Gene Flash, Syngene Bio Imaging).
14. (a) Primers for endoderm:
    Gata-4 [glutamyl-tRNA(Gln) amidotransferase subunit A-1] (571-bp amplicon size):
    - Forward: CTCCTACTCCAG CCCCTACC
    - Reverse: GTGGCATTGCTGGAGTTACC

    α-feto protein [alpha-1-fetoprotein] (299-bp amplicon size):
    - Forward: CCTTGGCTGCTCAGTACGACAAGG
    - Reverse: CCTGCAGACACTCCAGCGAGTTTC

    (b) Primers for mesoderm:
    Brac-T [brachyury transcription factor T-gene] (388-bp amplicon size):
    - Forward: AAGGAACCACCGGTCATCG
    - Reverse: CGTGTGCGTCAGTGGTGTGTAATG

    Flk-1 [fetal liver kinase-1] (599-bp amplicon size):
    - Forward: CCTGGTCAAACAGCTCATCA
    - Reverse: AAGCGTCTGCCTCAATCACT

    GSC [goosecoid] (516-bp amplicon size):
    - Forward: ATGCTGCCCTACATGAACGT
    - Reverse: CAGTCCTGGGCCTGTACATT

    (c) Primers for ectoderm:
    Sox-1 [sex determining region Y-box1] (388-bp amplicon size):
    - Forward: GCACACAGCGTTTTCTCGG

- Reverse: ACATCCGACTCCTCTTCCC

Nestin [neuroepithelial stem cell specific protein] (259-bp amplicon size):

- Forward: CGGCCCACGCATCCCCCATCC
- Reverse: AGCGGCCTTCCAATCTCTGTTCC

(d) Primers for hematopoietic progenitors:
Gata-2 [glutamyl-tRNA(Gln) amidotransferase subunit A-1] (336-bp amplicon size):

- Forward: TGCAACACACCACCCGATACC
- Reverse: CAATTTGGACAACAGGTGCCC

c-kit [cellular-homolog of protein kinase transmembrane receptor] (458-bp amplicon size):

- Forward: GCTCATAAATGGCATGCTCCAGTGT
- Reverse: GAAGTTGCGTCGGGTCTATGTAAAC

(e) Primers for myeloid-erythroid:
c-myb [cellular-myeloblastosis oncogene] (522-bp amplicon size):

- Forward: GAGCTTGTCCAGAAATATGGTCCTAAG
- Reverse: GGCTGCCGCAGCCGGCTGAGGGAC

SCL [stem cell leukemia/T-cell acute lymphocytic protein 1] (277-bp amplicon size):

- Forward: TAGCCTTAGCCAGCCGCTCG
- Reverse: GCGGAGGATCTCATTCTTGC

(f) Primers for erythroid:
Gata-1 [glutamyl-tRNA(Gln) amidotransferase subunit A-1] (581-bp amplicon size):

- Forward: ATGCCTGTAATCCCAGCACT
- Reverse: TCATGGTGGTAGCTGGTAGC

βH1-globin [hemoglobin beta-H1 chain] (610-bp amplicon size):

- Forward: TTTGCACACTTGAGATCATCTC
- Reverse: GGTCTCCTTGAGGTTGTTCCATG

β-major globin [beta-major globin] (610-bp amplicon size):

- Forward: ATGGTGCACCTGACTGATGCTG
- Reverse: GGTTTAGTGGTACTTGTGAGCC

(g) Housekeeping:
GAPDH [gene glyceraldehyde-3-phosphate dehydrogenase] (500-bp amplicon size):

- Forward primer: CATCACCATCTTCCAGGAGC
- Reverse primer: ATGCCAGTGAGCTTCCCGTC

15. Thermal cycler system (G-Storm, UK).

2.3 Western Blot Analysis

1. RIPA lysis buffer (Santa Cruz Biotechnology, Germany).
2. Protein Quantification: BCA™ Assay Kit (Pierce Biotechnology, UK).
3. Bovine serum albumin (BSA; Sigma-Aldrich, UK).
4. Tissue culture-grade 1× PBS without calcium or magnesium (Gibco Invitrogen Life Technologies, UK).
5. 96 well plate (VWR International, UK).
6. Enzyme-linked immunosorbent assay (ELISA) reader (ELx808, BIO-TEK, USA).
7. Laemmli sample buffer (contain 0.125 M Tris–HCL at pH 6.8, 4 % SDS, 20 % glycerol, 10 % βME, and 0.005 % bromophenol blue; BioRad, UK).
8. Precision Plus Protein™ All Blue Standards (BioRad, UK).
9. 10× Tris/Glycine premixed electrophoresis buffer (BioRad, UK).
10. NuPAGE® Novex® 4–12 % Bis-Tris Protein Gels, 1.0 mm, 10 well (Invitrogen, UK).
11. 0.2 μm nitrocellulose membrane (BioRad, UK).
12. C140 Mini Blot Module (Thermo Electron).
13. EC120 mini vertical gel electrophoresis unit (Thermo Electron).
14. SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology, UK).
15. Dyversity gel-documentation system (SynGene) using the Genesnap program (SynGene).
16. Rabbit polyclonal antibodies, GAPDH (1:20,000; Abcam, UK) and Gata-1 (1:200; Abcam, UK).
17. Goat anti-rabbit polyclonal IgG Horseradish peroxidase (HRP, 1:10,000; Abcam, UK).
18. 5 % nonfat skim milk solution.

2.4 Wright-Giemsa Analysis

1. Cytospin cyto-centrifudge set (Hettich Rotina 46R Centrifuge, Germany).
2. Wright-Giemsa stain (Sigma-Aldrich, UK).
3. Coplin staining jars (Sigma-Aldrich, UK).
4. Olympus BX51 microscope and DP50 camera (Olympus UK Ltd.).
5. Microscope slides (Thermo Scientific, UK).

3 Methods

3.1 Tissue Culture

3.1.1 Gelatin Coating of Culture Surface

1. Add adequate amount of 0.1 % gelatin solution to the culture surface to cover the entire surface of T75 flask and leave in a 37 °C incubator for at least 1 h.
2. Aspirate the gelatin solution before use and add the cell suspension.

3.1.2 Thawing Cells

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

3.1.3 Passaging Cells (Trypsinization)

1. Feed cells about 1 h before passaging.
2. Warm an aliquoted amount of Trypsin-EDTA 0.05 % (for mESCs culture) and 0.25 % (for HepG2 culture), and culture medium.
3. Aspirate medium from cultured cells and wash three times with 1× PBS.
4. Add Trypsin-EDTA solution and put cells in a 37 °C incubator for 5–10 min.
5. Gently pipet the cells up and down to separate them into single cells.
6. Transfer the cells into a centrifuge tube and add culture medium to the amount of passaging medium (*see* **Note 3**).
7. Centrifuge the cells for 5 min at 200 × *g*.
8. Remove the medium. Resuspend and plate out the cells required.

3.1.4 Freezing Cells

This freezing protocol is applicable to both mESCs and HepG2 cell lines.

1. Put freezing medium on ice.
2. Trypsinize the cells as described in Section 3.1.3.

3. Add culture medium to the cell-trypsin medium and centrifuge the cells at 200 × *g* for 5 min.
4. Remove the medium and resuspend cells in an appropriate volume of medium.
5. Count the cells and centrifuge as before.
6. Remove the medium and add an appropriate volume of the freezing medium so that the cell concentration is between 2×10^6 and 5×10^6 per milliliter of suspension.
7. Aliquot suspension into 1 mL cryovials, transfer the vials into a 100 % isopropyl alcohol bath, and leave the bath in the −80 °C freezer overnight. Alternatively, put them in a −20 °C freezer for 1 h, then transfer to a −80 °C freezer overnight.
8. Transfer to liquid nitrogen the next day.

3.1.5 Culture of HepG2 Cells and Preparation of HepG2 Conditioned Medium (CM)

1. Thaw a frozen cell vial (*see* Section 3.1.4) and resuspend cell in HepG2 cell medium (*see* Section 2.1.1).
2. Seed onto a non-gelatin-coated tissue culture flask.
3. Change the medium every 2 days and passage the cells every 3 or 4 days with 0.25 % Trypsin–EDTA (*see* Section 3.1.3).
4. For CM preparation, seed a density of 5.0×10^4 cell/cm^2 HepG2 cells on tissue culture flasks and culture the cells at 370 C in a 5 % CO_2 humidified water-based incubator for the following 4 days without medium changing.
5. Collect the culture medium after 4 days, filter-sterilized the medium using 0.22 μm filter unit (VWR International, UK).
6. Supplement the filtered medium with 0.1 mM β-ME and store the medium at −20 °C.
7. Prior to culture use, mix the medium collected from HepG2 cells as explained with fresh mESCs maintenance medium with a ratio of 1:1, and add 1,000 U/mL of LIF in the mixture (19) (*see* **Note 4**).

3.1.6 Culture of mESCs

Culture of Undifferentiated Cells

1. Seed undifferentiated mESCs on gelatin-coated flask and change the medium every other day.
2. When a subconfluent flask of undifferentiated mESCs is obtained, passage the cells by trypsinization (*see* Section 3.1.3) and plate the cells on gelatin-coated flask (Fig. 1).

Culture of Predifferentiated Cells with HepG2-CM ("CM-mESCs")

1. Passage the subconfluent flask of undifferentiated mESC, count and plate 3.0×10^6 cells per T-75 gelatin-coated flask.
2. Culture the cells in HepG2-CM (*see* Section 3.1.5) and change the medium every other day by adding 1,000 U/mL LIF (ESGRO) for 3 days.

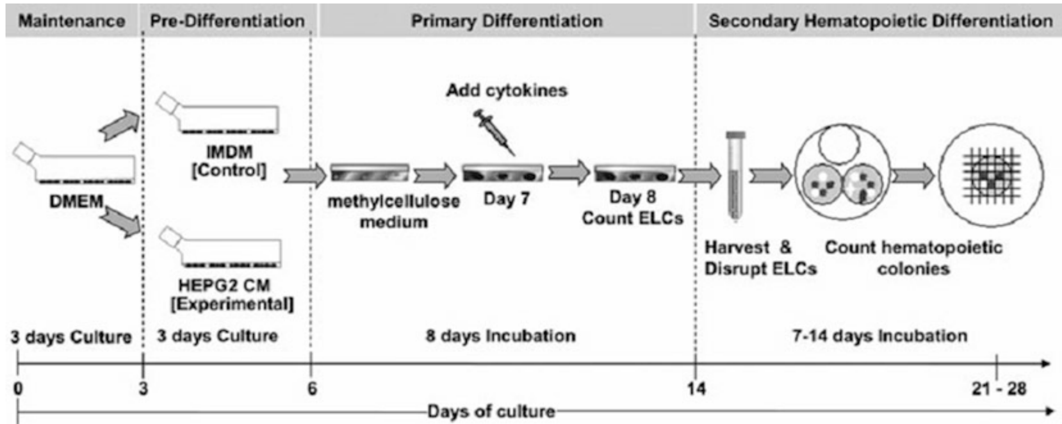

Fig. 1 Experimental design: control murine embryonic stem cell (mESC) and conditioned medium (CM)-mESC groups. The control group consisted of normal mESC culture, exposed to standard Iscove's modified Dulbecco's medium (IMDM) prior to differentiation that had undergone an 8-day embryoid-like colony (ELC) formation period using primary differentiation medium followed by a further 14 days in the secondary hematopoietic differentiation medium, as previously described (25). The experimental CM-mESC group consisted of mESCs previously exposed to HepG2 CM prior to differentiation, followed by the same 8-day ELC formation period using primary differentiation medium and a further 14 days of culture in the secondary hematopoietic differentiation medium, as explained previously. Both culture groups were analyzed simultaneously

Formation of "Embryoid-Like Colonies (ELCs)" in Primary Differentiation

1. Trypsinize the "CM-mESCs" and seed 3×10^3 cells/mL on low adherence 35-mm Petri-dishes supplemented with primary differentiation medium (*see* Section 2.1.1) (*see* **Note 5**).
2. On day 7 of culture, feed the cells with feeding medium (*see* Section 2.1.1) and culture for an additional day (day 8 of culture).

Hematopoietic Colony Forming Unit (CFU) Assay

1. Disrupt 8-day-old ELCs and replate 2.0×10^5 cells/mL on low adherence 35-mm Petri-dishes supplemented with hematopoietic differentiation medium (*see* Section 2.1.1) (*see* **Note 6**).
2. Incubate the cells for an additional 14 days. Score the resultant colonies using standard criteria based on the manufacturer's instruction (Stem Cell Technologies Hematopoietic Manual).

Figure 1 shows a schematic diagram of experimental design for conditioned medium mesodermal enhancement.

Total RNA isolations are adopted from Qiagen's instruction for Rneasy Kit product.

3.2 Reverse Transcriptase Polymerase Chain Reaction

3.2.1 Total RNA Isolation and Quantification

1. Harvest the cells by trypsinization as described in Section 3.1.3, neutralize the trypsin with serum-containing medium, and count the cells.
2. Centrifuge and discard the supernatant.
3. Wash the pellet with the ice-cold PBS and aliquot the cells into $<5 \times 10^6$.
4. Centrifuge and discard the supernatant.
5. Add 350 mL lysis buffer RLT for each 5×10^6 aliquot.
6. Mix by pipetting or vortexing (*see* **Note 7**).
7. Homogenize the cell lysate in a Qiashredder spin column and centrifuge at 14,000–16,000 × *g* for 2 min.
8. Add 350 mL 70 % ethanol into the cell lysate through the column.
9. Wash the total RNA that bound on the membrane column twice with buffer RW1 before ethanol removed using buffer RPE.
10. Elute the RNA in a 1.5 mL collection tube using RNase-free water.
11. Quantitate the RNA by spectrophotometry (*see* **Note 8**).
12. Store the RNA at −80 °C after quantification.

3.2.2 Complimentary DNA (cDNA) Formation by Reverse Transcriptase (RT)

Total mRNA is reversed transcribed into CDNA following Promega's instruction using Reverse Transcription System Kit.

1. Add 1 mg RNA sample in a PCR tube and adjust the volume to 10 mL by RNase-free water.
2. Heat the RNA sample at 70 °C for 10 min, briefly centrifuge and cool on ice for 5 min.
3. Prepare the master-mix by adding 5 mm $MgCl_2$, 1 mM of 2′-deoxynucleoside 5′-triphosphate (dNTP) mixture, 1 unit/μL recombinant RNasin ribonuclease inhibitor, 5 units/μL AMV reverse transcriptase and 0.5 mg/μL random primers.
4. Add the master mix (10 μL) to the RNA sample.
5. Incubate at 25 °C for 10 min, and then reverse transcriptase is performed at 42 °C for 40 min followed by denaturation at 90 °C for 5 min and a cooling step at 4 °C for 5 min.

3.2.3 Polymerase Chain Reaction (PCR) Formation

The data presented here are adopted from Invitrogen's protocol for the use of Taq polymerase and RT-PCR reagents.

1. Prepare an amount of 50 mL containing cDNA samples with mastermix (1× PCR Buffer, 0.2 mM dNTP mixture, 1.5 mM $MgCl_2$, 0.2 μM each primer (forward and reverse), 1 unit of Platinum Taq DNA Polymerase and DNase free water.
2. Adjust the temperature profile as follows: Taq polymerase activation, 95 °C for 2 min; denaturation, 94 °C for 30 s; annealing temperature (AT)for 30 s, 72 °C for 60 s; followed by final elongation step at 72 °C for 5 min.

3.2.4 Gel Electrophoresis

1. Mix 1.5 g of agarose agar powder with 100 mL of 1× TBE solution, dissolve in a microwave.
2. Add 10 μg/mL of EtBr into the mix solution before being casted to solidify.
3. Transfer the casted solidified gel to electrophoresis system filled with 1× TBE buffer.
4. Mix 5 μL of 100 bp DNA ladder and 1 μL of Blue/Orange loading dye used as DNA reference.
5. Load the DNA reference mixture into one well of the casted solidified gel.
6. Mix 10 μL of PCR sample and 2 μL of Blue/Orange loading dye. Load each sample mixture into each well of the gel.
7. Run electrophoresis at 95 V for 50 min.
8. Visualize the gel in a gel documentation system with ultra-violet light (Gene Flash, Syngene Bio Imaging).

The result of this experiment showed a high expression of mesodermal marker gene, Flk-1 in CM-mESCs than observed in control mESCs at day 8 of culture. Figure 2 shows the DNA gel electrophoresis of the PCR products.

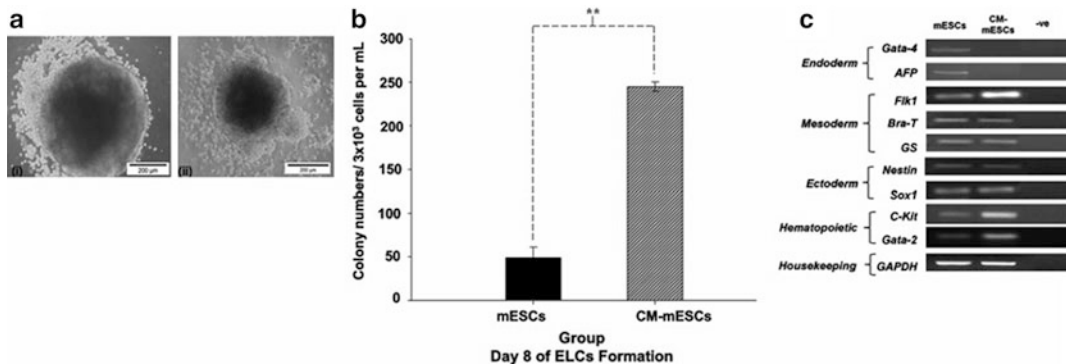

Fig. 2 Formation of ELCs in both culture groups at day 8. (**a**) Shown are examples of (i) control and (ii) CM-mESC ELCs. Images were captured at 20 · magnification under light microscopy (scales for each image: 200 mm). (**b**) A higher number of ELCs formed was observed in the CM-mESC group on day 8. Data shown are mean number of colonies—SD ($n = 5$; $^{**}p < 0.01$). (**c**) RT-PCR analysis of genes indicative of the three germ layers at day 8 of culture in control mESCs and CM-ESCs. Expression of genes of all three germ layers was observed in ELCs from control mESCs, whereas a higher expression of Flk-1 and loss of endoderm gene expression (GATA-4 and AFP) indicated more pronounced differentiation toward mesoderm with some early differentiation toward the hematopoietic lineage (Gata-2 and C-Kit) in those of CM-mESCs. Negative controls consisted of samples without cDNA. *AFP* a-fetoprotein, *Flk-1* fetal-liver kinase-1, *Bra-T* brachyury-T, *GS* goosecoid, *Sox-1* sry-related HMG box-1, *GAPDH* glyceraldehyde 3-phosphate dehydrogenase

3.3 Western Blot Analysis

3.3.1 Protein Extraction and Quantification

1. Harvest cell by scraping.
2. Lyse cell in 150 μL of RIPA lysis buffer, incubate at 4 °C for 30 min and centrifuge at 10,000 × *g* for 5 min.
3. Collect the protein-containing supernatant for quantification using BCA™ Assay Kit.
4. Dilute BSA in PBS from 0 to 500 μg/mL to create a protein standard curve.
5. Load 25 μL of sample into 96 well plate followed by mixing with 200 μL of BCA working reagent.
6. Incubate the plate in orbital shaking incubator at 37 °C for 30 min.
7. Take the reading at a wavelength of 560 nm on an ELISA microplate reader.
8. Quantify each protein sample against the BSA protein standard curve.

3.3.2 Sample Preparation and SDS-PAGE

1. Mix each protein sample with Laemli sample buffer, heat at 95 °C for 10 min and place on ice prior for gel loading.
2. Load 40 mg of protein mixed on NuPAGE® Novex® 4–12 % Bis-Tris Protein Gel electrophoresis.
3. Load protein ladder Precision Plus Protein™ All Blue Standards in one well.
4. Run one-dimensional SDS-PAGE to separate protein in EC120 mini vertical gel electrophoresis unit filled with 1× diluted Tris/Glycine premixed electrophoresis buffer.
5. Apply a constant voltage of 110 V at initial stage for 15 min or till the sample passes the stacking gel before a constant voltage of 150 V is apply for another 45 min or until the dye reached the bottom of the gel.
6. Transfer the protein blot onto 0.2 μm nitrocellulose membranes with 1× transfer buffer for 30 min followed by protein blotting on C140 Mini Blot Module at a constant current of 30 mA for 50 min (*see* **Note 9**).

3.3.3 Western Blot Analysis

1. Block nonspecific antibody binding by incubation with 5 % milk in 0.1 M PBS for 1 h.
2. Incubate blots at room temperature with primary and secondary antibodies for 1 h respectively.
3. Detect primary antibody binding with secondary antibody goat anti-rabbit polyclonal IgG Horseradish peroxidase by the SuperSignal West Pico chemiluminescent substrate.
4. Capture image using the Dyversity gel documentation system (SynGene) using the Gene snap program (SynGene).

3.4 Wright-Giemsa Cytospin Staining

1. Prepare 1×10^6 cells/mL cell suspension in 100 μL PBS and load it into a well of cytospin centrifuge set containing slide.
2. Spin the cells for 3 min at $100 \times g$.
3. Air-dry the slide for 5–10 min before stained with Wright-Giemsa stain in Coplin jar for 5–10 s.
4. Wash slide by dipping in deionised water for 2 min.
5. View image on the Olympus BX51 Microscope and capture using the DP50 camera.

The result of this experiment showed an early erythroid maturation identified by nucleated erythrocytes and macrophages found in the CFUs from CM-mESCs at day 7. This early erythroid maturation of adult type at day 7 of CM-mESC culture was confirmed by detection of Gata1 and β-major globin proteins with a lower expression of the primitive erythroid marker ζ-globin. Figure 3 shows Wright-Giemsa staining and the protein blotting of the western blot products.

4 Notes

1. 2-ME loses it thiol (-SH) group in the presence of metallic salts within 1–2 days Therefore, 2-ME must be added fresh to the culture medium immediately before use.
2. The duration and centrifugal force may differ for different cell lines, but generally 5 min at $200 \times g$ is suitable.
3. The serum in the culture medium inhibits trypsin.
4. HepG2 conditioned medium (CM) was formulated by mixing 50 % of fresh mESCs growth medium with 50 % of the medium collected from HepG2 cells and subsequently with addition of 1,000 units/mL of LIF in the mixture.
5. The cell density of the suspension will be cell line dependent and will vary on their ability to differentiate in methylcellulose. Optimally, there will be 50–100 colonies per dish in 1 mL of methylcellulose. As a first step, it may be necessary to perform a dose curve to determine the number of cells required to yield the optimal number of EBs. The number of EBs obtained should be linear with ESCs input.
6. The actual number plated will vary depending on the cell line and conditions used, as well the age of the colonies, but the density of $1–5 \times 10^4$ cells per dish should provide a useful starting range. When first establishing optimal plating densities, it is advisable to try two different cell concentrations which differ by two- to threefold.

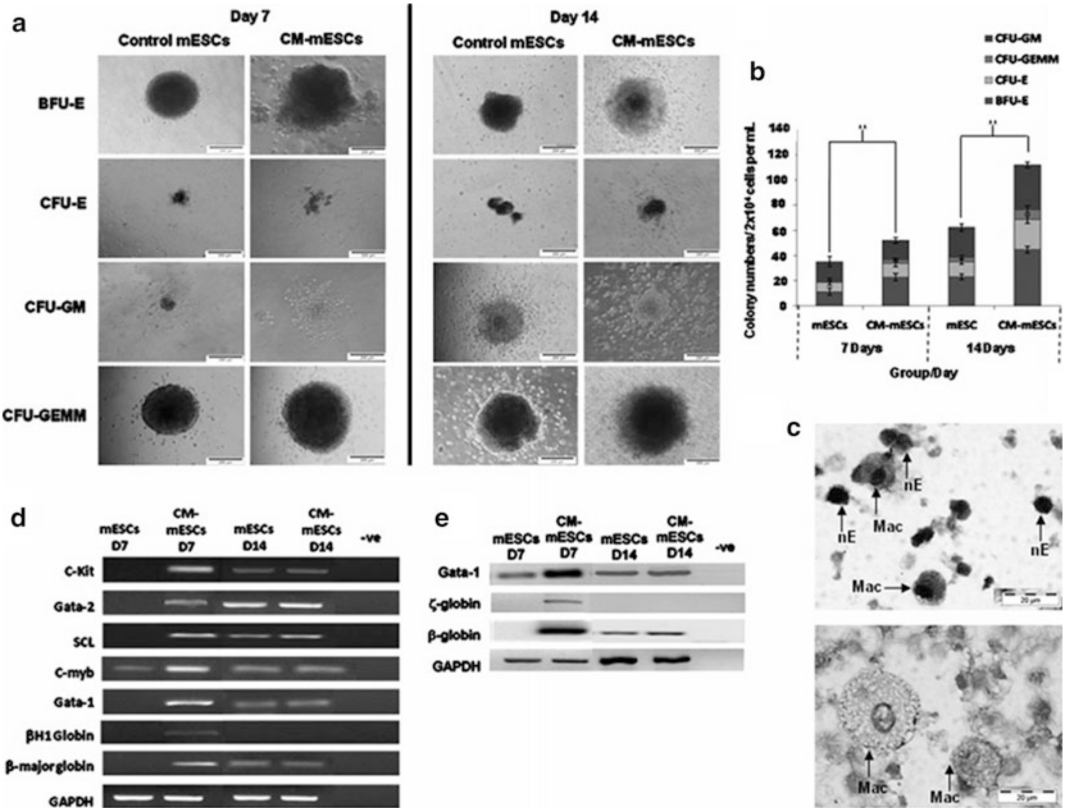

Fig. 3 Clonogenic capacity and gene and protein expression of terminally differentiated mESCs at days 7 and 14. (**a**) Standard methylcellulose assay was used to determine hematopoietic clonogenic capacity for burst-forming unit erythroid (BFU-E), colony forming unit (CFU)-E, CFU-GM, and CFU-GEMM at days 7 and 14 from ELCs of CM-mESCs and control mESCs. Morphological changes observed in the CM-ESC group at day 14 of culture were consistent with loss of hemoglobin within the colonies as indicated by the reduction of reddish hue. Images were captured at 20× magnification under light microscopy (scales for each image: 200 mm). (**b**) Summary bar graph of CFU numbers from both mESC groups at days 7 and 14 of the terminal differentiation culture. A higher number of all hematopoietic CFUs was observed in the CM-mESC group. (**c**) Wright-Giemsa staining of hand-picked BFU-E and CFU-GM colonies at day 7 of cultivated CM-mESCs confirmed the early maturation of hematopoietic colonies. Images were captured at 20× magnification under light microscopy (scales for each image: 200 mm). Data shown are mean number of colonies—SD ($n = 5$; $^{**}p < 0.01$). *nE* nucleated erythroid cells, *Mac* macrophage. (**d**) RT-PCR analysis of myeloid-erythroid genes in cells from day 7 CFU cultures in control mESCs and CM-mESCs indicates early expression of hematopoietic genes only in the experimental condition. Incubation for the standard 14 days resulted in expression of all hematopoietic and myeloid-erythroid markers in both control and experimental groups with loss of expression of the primitive erythroid marker bH1 globin. The negative control consisted of samples without cDNA. (**e**) Western blot analysis of CFUs from days 7 and 14 confirms early erythroid maturation at day 7 in the CM-mESC experimental group with primitive erythroid expression of z-globin protein. The negative control consisted of samples without protein extract

7. In the total RNA isolation protocol, the samples may be stored at −20 or −80 °C after homogenization in lysis buffer.
8. Find the OD at A_{260} and A_{280}. The A_{260}:A_{280} should between 1.6 and 2.
9. The blotted membrane was air-dried for at least 1 h at room temperature prior to immunostaining.

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Dopaminergic Differentiation of Human Embryonic Stem Cells on PA6-Derived Adipocytes

M. Oktar Guloglu and Anna Larsen

Abstract

Human embryonic stem cells (hESCs) are a promising source for cell replacement therapies. Parkinson's disease is one of the candidate diseases for the cell replacement therapy since the motor manifestations of the disease are associated with the loss of dopaminergic neurons in the substantia nigra pars compacta. Stromal cell-derived inducing activity (SDIA) is the most commonly used method for the dopaminergic differentiation of hESCs. This chapter describes a simple, reliable, and scalable dopaminergic induction method of hESCs using PA6-derived adipocytes. Coculturing hESCs with PA6-derived adipocytes markedly reduces the variable outcomes among experiments. Moreover, the colony differentiation step of this method can also be used for the dopaminergic induction of mouse embryonic stem cells and NTERA2 cells as well.

Keywords: Parkinson's disease, Human embryonic stem cells, SDIA, Adipocytes, Dopaminergic differentiation

1 Introduction

With the recent findings of somatic cell nuclear transfer in humans, human embryonic stem cells (hESCs) become a promising "personalized" source of cells for regenerative medicine (1). During the past decade, several groups have established various methods for differentiating hESCs into dopaminergic (DAergic) neurons (2–5). The most commonly used method for the DAergic differentiation of hESCs is the stromal cell derived inducing activity (SDIA); however, there is a significant variability for the SDIA of PA6 cells among and within the PA6 batches (6, 7). We revealed that adipocytes within the heterogeneous PA6 population robustly express all previously claimed factors eliciting SDIA (6, 8–10). Moreover, PA6-derived adipocytes also express GDNF, BDNF, and PEDF that have been previously shown to promote dopaminergic neuron differentiation and survival. Therefore, it is reasonable to use the PA6-derived adipocytes as a more homogenous population of feeder cells to induce the DAergic differentiation of hESCs.

In this chapter, we describe a fast, simple, and reliable method for differentiating hESCs into dopaminergic neurons

using PA6-derived adipocytes as feeder cells. The low variability in the outcomes of the DAergic differentiation in this method allows a more accurate platform for further studying the DAergic differentiation mechanisms. Our method can also be used for differentiating mouse embryonic stem cells (mESCs) and NTERA2 cells into dopaminergic neurons, which allows comparison studies in DAergic differentiation. Moreover, we describe a highly scalable method for generating neural progenitors (NPs). Our method is suitable for generating NPs for large-scale applications and monolayer differentiation. These hESC-derived NPs can also be frozen and stored for long times, which facilitates the planning and execution of large-scale assays. Our method includes three simple steps for DAergic differentiation: Coculturing hESCs on PA6-derived adipocytes, expansion of NPs and monolayer differentiation.

2 Materials

2.1 Equipment

1. Cell culture disposables.
2. Laminar flow hood with HEPA filter.
3. Fume hood.
4. CO_2 incubator with humidity and temperature control.
5. Refrigerated cell culture centrifuge.
6. Inverted phase-contrast microscope.
7. Hemocytometer.
8. 37 °C water bath.
9. Mr. Frosty 1 °C freezing container (Thermo Scientific/Nalgene).

2.2 Cell Culture

1. PA6 maintenance medium: MEM-α medium (PAA, Austria) with 10 % FBS (Invitrogen).
2. Adipogenic differentiation medium: MEM-α medium (PAA, Austria) with 10 % FBS (Invitrogen) + 0.25 μM Dexamethasone (Thermo Fisher) + 0.5 mM 3-isobutyl-1-methylxanthine (Thermo Fisher).
3. Coculture (CC) medium: Glasgow's modified Eagle's medium (GMEM) + 8 % KnockOut serum replacement (KSR) + 2 mM GlutaMAX, 0.1 mM nonessential amino acids (NEAA), 1 mM pyruvate, 0.1 mM 2-mercaptoethanol (2-ME) (All from Invitrogen).
4. NS medium: Neurobasal medium (Invitrogen) + 0.5 mM GlutaMAX (Invitrogen) + 0.01 % heparin (Sigma-Aldrich).
5. Freezing medium for NPs: Conditioned NS medium + 20 ng/ml bFGF + 20 ng/ml EGF + 10 % DMSO.
6. Accutase (Invitrogen).

7. Trypsin (Invitrogen).
8. Poly-L-lysine (Sigma-Aldrich).
9. Laminin (R&D systems).
10. bFGF (Invitrogen).
11. Y-27632 dihydrochloride (Tocris).

2.3 Adipocyte Staining

1. Stock solution: 300 mg Oil Red O powder (Fisher) in 100 ml 99 % isopropyl alcohol.
2. Working solution: three parts Oil Red O stock solution mixed with two parts distilled water and filtered through Whatman paper (Fisher) (*see* **Note 1**).
3. Hematoxylin (Fisher).
4. 10 % formalin solution (Fisher).

3 Methods

3.1 Adipogenic Differentiation of PA6 Cells

1. Culture PA6 cells until total confluency.
2. Replace with fresh PA6 maintenance medium and continue culturing the cells in total confluency for an additional 2 days.
3. On the third day, replace with fresh adipogenic differentiation medium to induce differentiation. Change this induction medium every other day, and culture the cells in this medium for 4 days.
4. On the seventh day, replace the adipogenic differentiation medium with PA6 maintenance medium and culture the cells for an additional 4 days (*see* **Note 2**).

3.2 Oil Red O Staining

All steps involving formalin and isopropanol should be performed in a fume hood.

1. Remove the medium and rinse the cultures with DPBS.
2. Remove DPBS and fix the cells with 10 % formalin for 30 min at room temperature.
3. Rinse the culture with distilled water and incubate in 60 % isopropanol solution for 5 min.
4. Add freshly prepared Oil Red O working solution and incubate for 5 min at room temperature.
5. Rinse the cultures with tap water at room temperature until the water rinses off clear.
6. (Optional) For counterstaining, add hematoxylin and incubate for 1 min at room temperature.
7. (Optional) Remove the hematoxylin and rinse the culture with room temperature tap water until the water rinses off clear.

8. Add tap water to cultures and observe under phase-contrast microscope.

3.3 Conditioning Medium with PA6-Derived Adipocytes

1. Remove the PA6 medium and rinse the PA6-derived adipocyte culture with DPBS twice.
2. Add NS medium (12 ml for T75 flask) to the culture and incubate for 24 h.
3. Collect the medium and centrifuge at 500 × *g* for 5 min to exclude the debris.
4. (Optional) By using a 0.22 μM hydrophilic PVDF syringe filter, sterilize the conditioned medium and remove any remaining cells.
5. If not used immediately, freeze and store the conditioned medium at −20 °C (*see* **Note 3**).
6. Discard the culture (*see* **Note 4**).

3.4 Plating PA6-Derived Adipocytes as Feeder Cells

1. Coating the dishes. Coat culture dishes (or culture slides) with 0.1 % gelatin and incubate at least for 30 min at room temperature before plating the feeder cells.
2. Single cell dissociation. Rinse the culture with DPBS twice. Add 0.05 % trypsin-EDTA (3 ml for T75 flask) and incubate at 37 °C for 5 min. Tap the flask few times from the sides and the bottom to detach the cells, and dissociate into single cells by pipetting several times. Add PA6 maintenance medium to stop the enzymatic reaction. Centrifuge at 300 × *g* for 5 min at 4 °C. Carefully discard the PA6 medium and resuspend the pellet in CC medium (*see* above). Count the cells with a hemocytometer.
3. Plating cells. Aspirate the gelatin and wash the gelatin-coated dishes with DPBS once. Plate 80,000 cells/cm^2 (*see* **Note 5**). Incubate the feeder cells in the incubator for 2 days at 37 °C and 5 % CO_2.

3.5 Colony Differentiation

1. Dissociation of the cells. The day before dissociation (Day-1) add 10 μM ROCK-inhibitor (Y-27632 dihydrochloride) to the hESC culture to increase cell survival during the dissociation process. Depending on the hESC line used, dissociate hESCs either enzymatically or mechanically (*see* **Note 6**). If the hESCs will be dissociated mechanically, replace the hESC maintenance medium with CC medium just before cutting the colonies into small clumps. Use a 23 G needle or commercially available stem cell cutting tools to cut hESC colonies into small clumps (10–20 cells per clump) (Fig. 1c). Plate the dissociated small clumps on feeder cells. Only at the first day of coculture, add 4 ng/ml bFGF to the medium to improve cell survival.
2. Maintenance. Culture the cells in the incubator at 37 °C and 5 % CO_2. Replace half of the medium carefully every other day

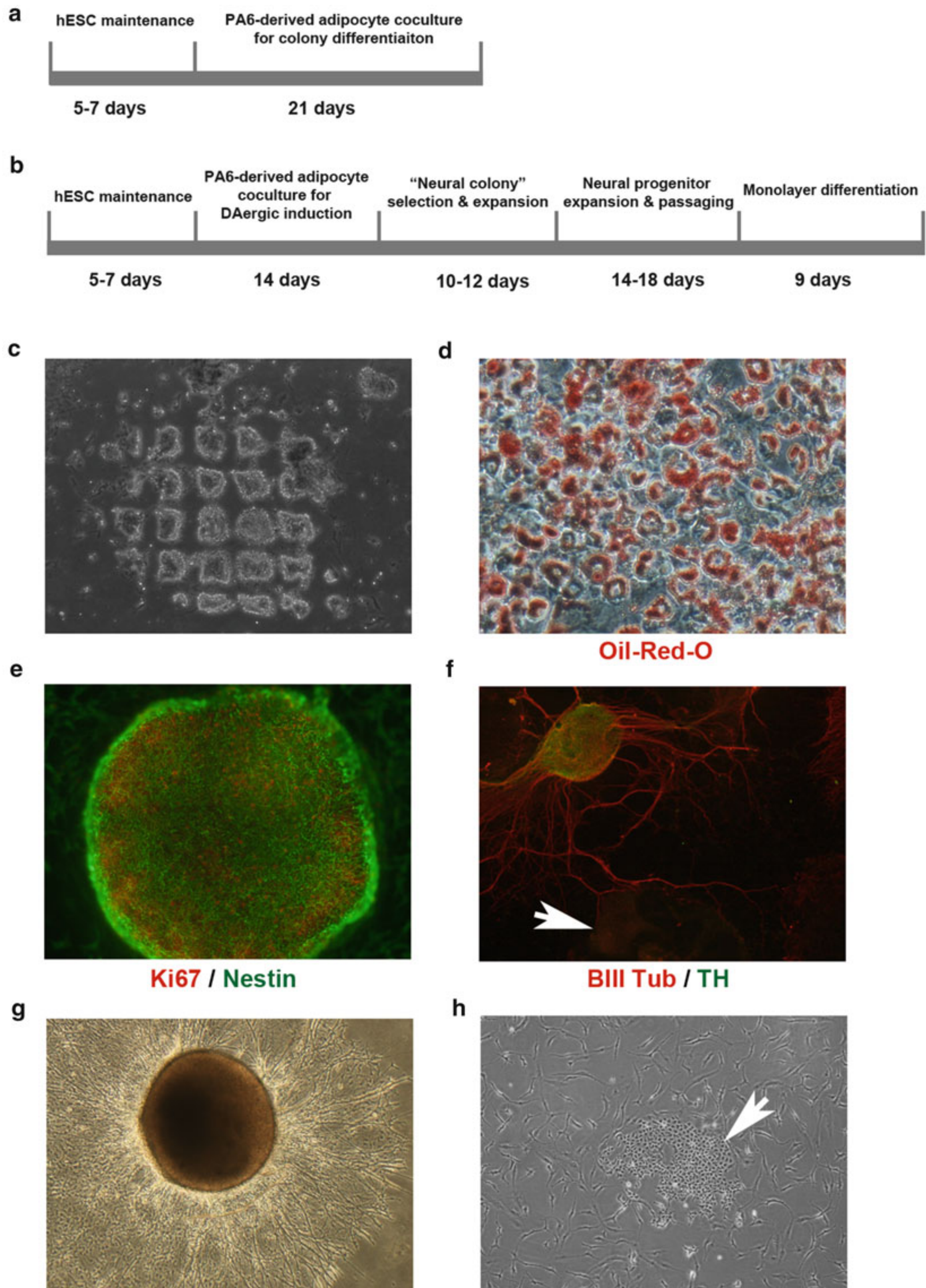

Fig. 1 Dopaminergic differentiation of hESCs. (**a**) Coculturing hESCs on PA6-derived adipocytes gives rise to dopaminergic colonies in 3 weeks (**b**) Three-step protocol requires the selection of neural colonies on day 14 of coculture, expansion of neural progenitors, and monolayer differentiation (c) Undifferentiated hESC colonies

without exposing the colonies to air. For neural progenitor generation and monolayer differentiation, transfer the "neural colonies" on day 14 as described below. For full colony differentiation continue until day 21 (*see* **Note 7**).

3.6 Monolayer Neural Progenitor Generation and Differentiation

1. Detach the "neural colonies" on day 14 (*see* **Note 8**) using a 23 G needle under a microscope.
2. Plating. Collect the detached colonies using a 100 ml micropipette and plate on a gelatin coated 35 mm dish.
3. Maintenance. Use NS medium conditioned with PA6-derived adipocytes for culturing. Freshly add 10 ng/ml of bFGF and 2 % B27 (without retinoic acid) to the medium. Replace half the medium every other day.
4. Passaging. Within few days, cells from the attached colonies spread outward (Fig. 1h). Any colonies displaying morphological difference than the neural colonies at this stage should be removed using a micropipette. When the cells become confluent (typically in 10–14 days), aspirate the medium and wash the cells once with PBS. Add 0.4 ml Accutase and incubate for 5 min at 37 °C and 5 % CO_2. Gently triturate to single cells using a 1 ml pipette. Add NS medium and centrifuge at 300 × *g* for 5 min at 4 °C. After centrifugation resuspend the cells in conditioned NS medium with 10 ng/ml of bFGF and 2 % B27 (without retinoic acid). Plate the cells (5×10^4 cells/cm^2) onto a new gelatin coated dish. From the second passage and on, the neural progenitors can be split at a 1:3 ratio using the same procedure (*see* **Note 9**). The neural progenitors at this stage can be frozen, used for further analyses or differentiated (*see* **Note 10**).
5. Monolayer DAergic differentiation. Plate NPs on glass chamber slides (or plastic culture dishes) coated with poly-L-lysine and laminin (*see* below) at the density of 100,000 cells/cm^2. Use CC medium at this stage. To increase cell survival, add 4 ng/ml of bFGF to the medium just for the first day. To induce terminal differentiation, culture the cells in the absence

Fig. 1 (continued) are mechanically cut into small clusters for coculturing (**d**) Oil Red O staining of PA6-derived-adipocytes confirms the successful adipogenic differentiation (**e**) "Neural colonies" have a round, dome shape in the culture. Immunostaining results of the "neural colonies" on day 14 demonstrate that those colonies are rich in nestin expressing cells. Some cells also express the proliferation marker Ki67 (**f**) On day 21 of coculture, neural colonies with round shape robustly express TH and BIII-tubulin markers whereas other types of colonies (*arrowhead*) poorly express those markers (**g**) When the "neural colonies" are selected on day 14 and plated on gelatin coated dishes, neural progenitors start to spread outward (**h**) During NP expansion, morphologically distinct two types of cells emerge. First, flat shaped cells emerge and start to cover the surface. Later, neural progenitors start to emerge as clusters (*arrowhead*) on top of these cells

of bFGF afterwards. Perform half medium change every other day. Culture the cells until day 9.

3.7 Poly-L-Lysine and Laminin Coating

1. Dilute Poly-L-lysine (PLL) with DPBS at 1:2 ratio.
2. Cover the culture dish or slide with this diluted PLL and incubate at 37 °C overnight.
3. Remove the PLL and wash the culture slide with DPBS once.
4. Cover the culture slide with 10 μg/ml laminin and incubate at 37 °C overnight.
5. Wash the culture slide with DPBS once before use.

3.8 Freezing and Storing Neural Progenitors

1. Follow the steps described above for passaging.
2. After centrifugation suspend the cells in freezing medium.
3. Count the cell number using a hematocytometer.
4. Distribute the cells into cryotubes.
5. Put the cells into −80 °C freezer overnight in a 1 °C freezing container.
6. Transfer the cells into −150 °C next day.

3.9 Thawing Neural Progenitors

1. Warm up 9 ml NS medium to rinse.
2. Thaw the vial in 37 °C water.
3. Add 9 ml pre-warmed NS medium and spin down at 500 × *g* for 5 min.
4. Suspend the pellet in conditioned NS medium and plate on 0.1 % gelatin-coated dishes.

4 Notes

1. Although the stock Oil Red O solution can be stored up to 1 year at room temperature, working solution is only stable for 2 h. Therefore, working solution should be freshly prepared just before staining.
2. Adipogenic differentiation protocol for PA6 cells slightly differs from the protocols for other preadipocyte cell lines. It does not require the addition of insulin or PPARγ (11). In our hands, we could differentiate PA6 cells into adipocytes with 81 % efficiency; however, there might exist variations between batches. It should also be noted that PA6 cells in the culture do not differentiate homogeneously at the same pace. Thus, not only mature adipocytes with big lipid vesicles, but also “less-mature” adipocytes with smaller lipid vesicles exist in the culture at the end of the differentiation (Fig. 1d). To determine the differentiation efficiency accurately, Oil Red O staining is recommended.

3. The conditioned medium can be stored at −20 for up to 1 year.
4. Each PA6-derived adipocyte culture can only be used once for conditioning medium.
5. Not all the PA6 cells become adipocytes at the end of the differentiation. If the differentiated PA6 culture is plated with low numbers of cells, undifferentiated PA6 cells start to proliferate, increasing their percentage in the culture. Therefore, high numbers of cells are plated in order to stop proliferation of the undifferentiated PA6 cells by contact inhibition (Fig. 1d).
6. If the hESCs are enzymatically dissociated, plate 250 hESCs/cm^2 suspended in CC medium. When calculating the number of hESCs after enzymatic dissociation, be aware of the number of the feeder cells in the suspension if hESCs are maintained on feeder cells. Subtract the number of feeder cells when calculating the exact number of hESCs to be plated.
7. Our results revealed that not only hESCs but also mESCs and NTERA2 cells can be differentiated into DAergic neurons on PA6-derived adipocyte coculture (Fig. 2). Similar to hESCs, 21 days of differentiation is required for NTERA2 cells, whereas 14 days of culturing is sufficient for mESCs.
8. Similar to PA6 coculture, morphologically different types of colonies are observed on the 14th day of PA6-derived adipocyte coculture (7, 12). Among those colonies, "neural colonies" that are rich in neural cells are identified according to a series of morphological criteria: They have a round shape and brownish color, often extend short processes outside the core of the colony, and are easily detached from the feeder cells using a 23 G needle (Fig. 1e–g). Other colony types such as the ones having flat, amorphous or cystic morphology do not have the required neural identities (Fig. 1f).
9. After each passage, two morphologically distinct cell types emerge in the NP culture dish. Large, flat-like cells resembling astrocytes and small, round-shaped, dark colored cells with

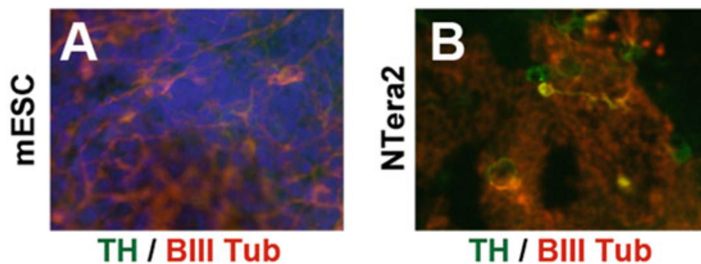

Fig. 2 DAergic inducing activity of PA6-derived adipocytes on different cell types. Coculturing on PA6-derived adipocytes promotes the DAergic differentiation of (**a**) mESCs and (**b**) NTERA2 cells

high nucleus-to-cytoplasm ratio. Our ICC results revealed that the small cells are the neural progenitors. The large, flat cells initially form a monolayer occupying a large proportion of the culture dish; however, this is not the time point to passage cells. On top of this monolayer, small round-shaped cells (NPs) start to emerge as clusters (Fig. 1h). When those neural progenitors, cover more than half of the culture dish, the cells can be passaged. It should also be noted that coculturing hESCs with PA6-derived adipocytes significantly reduces the percent of Ki67-positive NPs generated compared to NPs generated with PA6-coculture (25 % vs. 59 %, $p < 0.002$). Therefore, in this protocol NPs have low proliferation rate and reach to passaging confluency in 14–18 days.

10. The NPs generated can be expanded several passages without losing their differentiation capacity. They can also be frozen and stored for long periods. Following cryopreservation, typically ½ to 1/3 of the plated cells survive. We usually plate 1 M cells on 35 mm culture dish, 2.5 M cells on T25 flasks and 6 M cells on T75 flasks after thawing.

Acknowledgements

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Intracellular Calcium Measurements for Functional Characterization of Neuronal Phenotypes

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Abstract

The central and peripheral nervous system is built by a network of many different neuronal phenotypes together with glial and other supporting cells. The repertoire of expressed receptors and secreted neurotransmitters and neuromodulators are unique for each single neuron leading to intracellular signaling cascades, many of them involving intracellular calcium signaling. Here we suggest the use of calcium signaling analysis upon specific agonist application to reliably identify neuronal phenotypes, being important not only for basic science, but also providing a reliable tool for functional characterization of cells prior to transplantation. Calcium imaging provides various cellular information including signaling amplitudes, cell localization, duration, and frequency. Microfluorimetry reveals a signal summarizing the entire population, and its use is indicated for high-throughput screening purposes.

Keywords: Neuron, Differentiation, Stem cell, Calcium signaling, Phenotype, Neuronal receptors

1 Introduction

The number of studies on the mechanisms of neural differentiation of embryonic stem cells (ESC), induced embryonic stem cells (iPSC), and their therapeutic potential in neurobiology, neuropathology, pharmaceutical research, and cellular therapy fields is increasing daily [1]. After establishment of neural lineage commitment of pluripotent ESC in vitro, a variety of differentiation protocols emerged trying to achieve greater efficiency and specificity. The majority of these protocols consist of the spontaneous origination of embryoid bodies (EB) by cultivating ESC in a feeder- and serum-free medium following supplementation with growth factors and other agents that induce neural differentiation [2].

Aiming at a homogeneous population at the endpoint of differentiation, an important challenge lies in the phenotypic characterization of possible intermediate cell types and neurons. Along differentiation, ESCs lose pluripotent markers while they gain specific lineage markers [3]. These changes can be tracked by immunocytochemistry, flow cytometry, and polymerase chain reaction among other methodological approaches. However, all of these

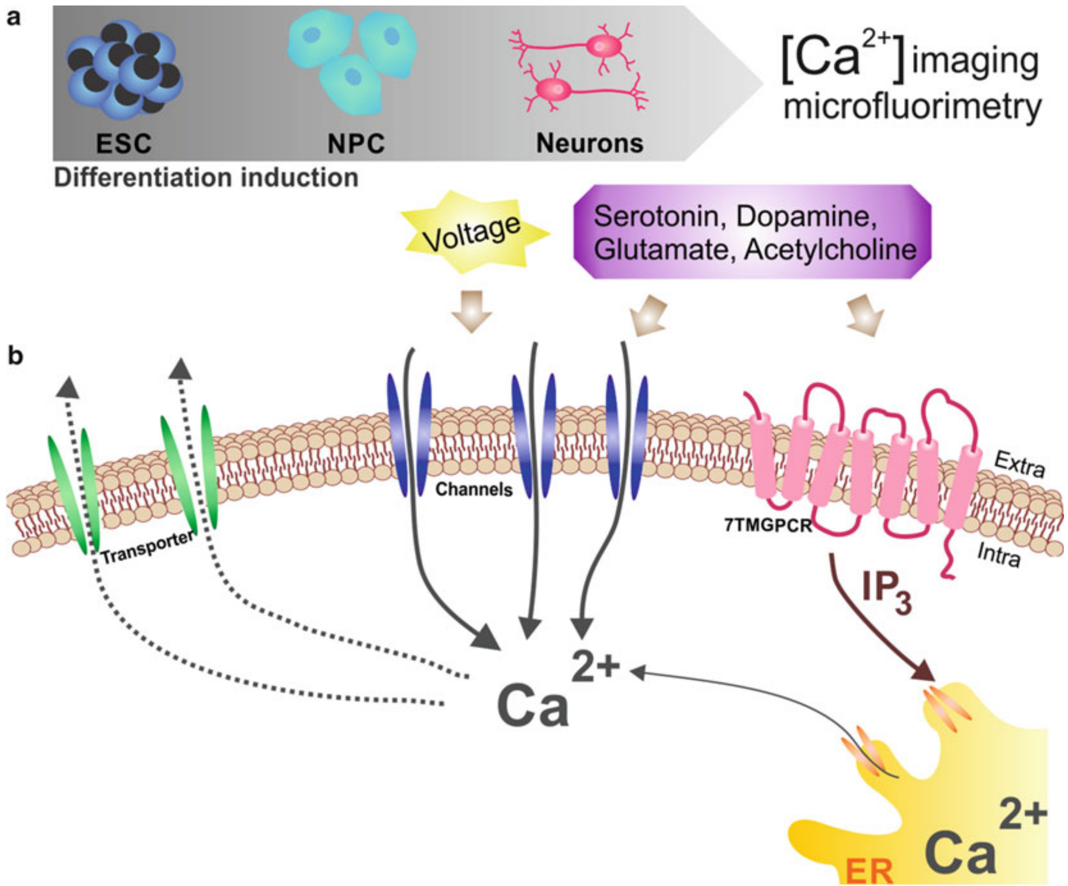

Fig. 1 Neuronal differentiation and calcium signaling in neurons. Depending on the neuronal phenotype obtained during neuronal differentiation, cells may be responsive to one or many neurotransmitters triggering intracellular calcium signaling pathways. (**a**) After neuronal differentiation induction, cells differentiate into different types of neurons and can be characterized by calcium imaging or calcium microfluorimetry. (**b**) Voltage-gated calcium channels can be triggered during membrane depolarization resulting in extracellular calcium entry. This can be achieved by adding 60 mM KCl. Furthermore, some neurotransmitters like serotonin, glutamate, acetylcholine, and dopamine can increase intracellular calcium concentration by activating ion channels or seven-transmembrane G protein-coupled receptors (7TMGPCRs), by formation of inositol 1,4,5-trisphosphate (IP3) and consequently extrusion of calcium from the endoplasmic reticulum

methods verify the expression of some protein markers without evaluation of functional cellular activity.

Detailed information about the neuron phenotype can be obtained by its functional expression of neurotransmitters and their functional receptors (Fig. 1) using calcium imaging or microfluorimetry for measuring cytosolic calcium concentration during activation of these receptors after applying agonist/stimuli. Functional analysis of receptors confirms viability and functionality of differentiated cells, thus increasing reliability and reproducibility of differentiation protocols.

Cellular calcium signaling is a ubiquitous and extremely versatile system, once there are many ways to combine the molecular repertory for creating numerous signals with many temporal, spatial and calcium concentration level patterns [4–6]. At rest, many neurons reveal cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) of 50–100 nM, and upon stimuli these rise up to 100 times higher [4]. Transient oscillations of intracellular $[Ca^{2+}]_i$, called intracellular calcium transients ($\Delta[Ca^{2+}]_i$), induce changes of expression and activity of several proteins, triggering many cellular processes including proliferation and differentiation [7]. Neurons have a highly developed calcium signaling system that is involved in neurotransmitter release, synaptic transmission, cerebral rhythms, memory formation, and learning [8, 9].

Patterns of $\Delta[Ca^{2+}]_i$ change during neuronal differentiation according to timing and to neuronal phenotype in development [10–13]. Functional studies with neural lineages have shown calcium cascade signaling mediated by cholinergic, glutamatergic, serotonergic, dopaminergic and purinergic receptors [14, 15].

Currently, there are many technologies available for calcium imaging analysis. Since the first appearance of calcium-binding bioluminescent proteins and fluorescent probes, development of new approaches in this area allowed detailed investigation of calcium signaling function [15]. The main rationale is to follow $[Ca^{2+}]_i$ levels through time using a calcium indicator that emits a fluorescence signal correlated to calcium concentration with an imaging microscope or fluorescence-microplate reader.

In this chapter, we describe briefly a protocol of pan-neural differentiation and two methods for intracellular calcium measurement commonly used for helping to monitor differentiation process of ESCs and final obtained phenotypes.

2 Materials

Prepare and store all reagents at 4 °C unless stated differently. All solutions should be prepared using ultrapure water and analytical grade reagents. All reagents and materials must be sterile.

2.1 Pan-Neural Differentiation of Feeder-Free Embryonic Stem Cells

2.1.1 Culture of Feeder-Free ES Cells

PBS 1×: Phosphate buffer saline (137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4). Dissolve the reagents listed above in water. Adjust the pH to 7.4 with HCl. Dispense the solution into aliquots and sterilize them by autoclaving.

Gelatin from porcine skin solution: 0.2 % gelatin in PBS 1×. pH 7.4. Sterilize by autoclaving and store at 4 °C.

ES medium: Glasgow's modified Eagle medium or Dulbecco's modified Eagle medium high glucose, 15 % fetal bovine serum ES-QUALIFIED tested, 1× non-essential amino acids

(NEAA), 1 mM sodium pyruvate, 1000 U/ml ESGRO LIF. pH 7.4. Sterilize by filtering through 0.22 μm pore size membranes.

Trypsin solution: 0.25 % trypsin 1 mM EDTA (ethylenediaminetetraacetic acid). pH 7.4. Sterilize by filtering with 0.22 μm pore membrane.

EB differentiation medium: Glasgow's modified Eagle medium or Dulbecco's modified Eagle medium high glucose, 20 % fetal bovine serum, 1× NEAA, 1 mM sodium pyruvate. pH 7.4. Sterilize by filtering through 0.22 μm pore membranes.

Neurobasal medium: Dulbecco's modified Eagle medium/nutrient mixture F-12, 1 % Bottenstein's N-2 formulation, 100 ng/ml b-FGF.

2.2 Calcium Imaging

2.2.1 Calcium Indicator Incorporation

Staining buffer: Dulbecco's modified Eagle medium/nutrient mixture F-12 or extracellular medium without magnesium (140 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 10 mM glucose, pH 7.4), 5 μM Fluo-3 AM, 10 μl Me_2SO, 0.1 % pluronic surfactant F-127.

2.2.2 Recording Data

Time-lapse buffer: Dulbecco's modified Eagle medium/nutrient mixture F-12.

2.2.3 Equipment

Hardware: Inverted Research Microscope ECLIPSE-TiS (Nikon, Melville, NY) equipped with a 14 bit high-resolution CCD camera CoolSNAP HQ2 (Photometrics, Tucson, AZ).

Software: NIS-Elements Advanced Research (Nikon).

2.3 Microfluorimetry

2.3.1 Calcium Indicator Incorporation

Probenecid Stock Solution: Probenecid, 1 N NaOH, "Component B" buffer from FlexStation Calcium Assay Kit (Molecular Devices Corp.). To prepare 250 mM probenecid stock solution, mix properly until completely dissolved 77 mg probenecid with 500 μl of 1 N NaOH. Add 500 μl of "Component B" buffer from "FlexStation Calcium Assay Kit." Mix it properly again.

Component B buffer with probenecid: 1000 μl 250 mM Probenecid stock solution, "Component B" buffer of the FlexStation Calcium Assay Kit (Molecular Devices Corp.). Mix properly. This buffer can be used to make both your dye loading buffer and agonist dilutions.

Staining buffer: FlexStation Calcium 4 Assay Kit (Molecular Devices Corp.) containing 2.5 mM probenecid.

Dish: 96-well black microplate with clear bottom.

2.3.2 Equipment

Hardware: FlexStation 3 (Molecular devices Corp.).

Software: Softmax Pro (Molecular devices Corp.).

3 Methods

3.1 Pan-Neural Differentiation of Feeder-Free Embryonic Stem Cells

All reagents and materials must be sterile.

3.1.1 Culture of Feeder-Free ES Cells

E14tg2a cell line: 129/Ola-derived HPRT-negative ES cells (**Note 1**).

- *Gelatin coating*

  All dishes, flasks, and plates should be gelatinized before use, if not otherwise mentioned.

  1. Add gelatin from porcine skin solution.
  2. Keep for 10 min at room temperature.
  3. Aspirate thoroughly.

- *Splitting cells*

  2 days after seeding with ESC medium or when cells reach 80 % density confluence.

  1. Aspirate medium and wash cells with PBS 1× once (Fig. 2a).
  2. Add 1 ml trypsin solution.
  3. Incubate at 37 °C for 3 min.
  4. Add 3 ml of ESC medium.

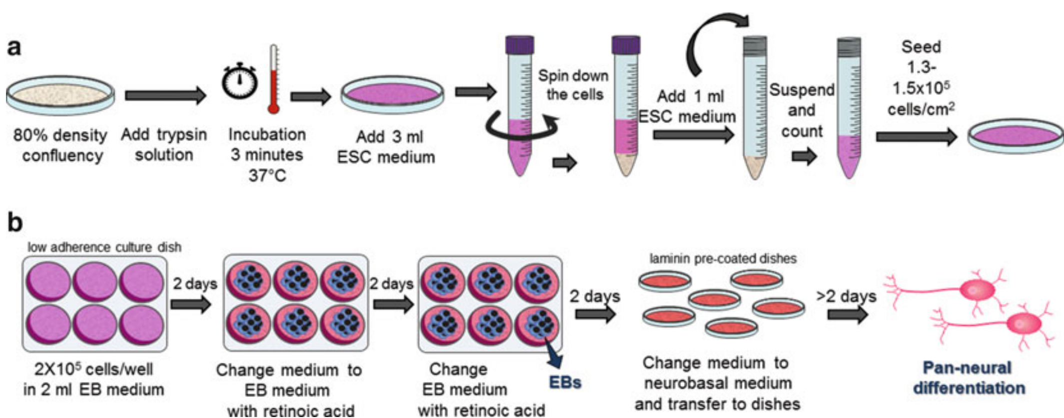

Fig. 2 Illustration of the embryonic stem cell maintenance (**a**) and neuronal differentiation (**b**) protocols (**Note 11**)

Table 1
Instructions for seeding cells

Seeding cell numbers	Flask/dish size	Medium volume
3–4 × 10^5	25 cm^2 small flask	5 ml medium
1 × 10^6	75 cm^2 middle flask	15 ml medium
2 × 10^6	150 cm^2 large flask	25 ml medium
1 × 10^5	60 mm dish	4 ml medium
5 × 10^5	90 mm dish	10 ml medium

5. Spin down the cells.
6. Discard the supernadant and suspend cells in 1 ml ES medium.
7. Count the number of cells.
8. Seed 1.3–1.5 × 10^5 cells/cm^2 (Table 1).
9. Number of cells usually reach 8–10 times higher confluence than seeded ones do.

- *Differentiation*

1. Seed ~2 × 10^5 cells/well in 2 ml EB differentiation medium using 6-well low adherent culture dishes for induction of EB formation (Fig. 2b).
2. After 2 days of floating culture, sediment cells in a 15 ml conical tube, replace EB differentiation medium, and add 5 μM all-trans retinoic acid previously dissolved in DMSO (**Notes 2** and **3**).
3. After 2 more days of culture repeat **step 2**.
4. Following 2 further days sediment cells, replace EB medium by neurobasal medium, and seed EBs into laminin pre-coated dishes (as described below).
5. Replace neurobasal medium for a fresh one each 2 days until the cells are finally differentiated to neurons (after 10 days).
6. Neurons are stable for more than 30 days in in vitro culture.

- *Laminin coating*

1. Add 50 μg/ml laminin to dish, and incubate 37 °C for at least 30 min.
2. Wash 3× with 1×PBS.
3. Dishes can be reused many times.

3.2 Calcium Imaging

3.2.1 Calcium Indicator Incorporation

1. After 8, 16 or 20 days since the first medium change to neurobasal medium (item IV of the above-described differentiation method), incubate EB for 45 min in staining buffer at 37 °C in cell culture incubator (water-saturated atmosphere, 5 % CO_2) (**Note 4**).
2. Wash cells twice with time-lapse buffer and add 2 ml of this time-lapse buffer for subsequent calcium measurements.
3. Keep them in time-lapse buffer for 20 min to obtain complete deesterification of the acetoximethylester group (AM) of the fluorophore.

3.2.2 Live Imaging

1. Set parameters at Nis elements AR software for time-lapse measurements at *6D measurement* icon. Adjust to 2 frames/second and select FITC-band-pass filter.
2. Image cells for at least 5 s for basal fluorescence.
3. Apply 10× concentrated agonist in 200 μl volume while recording data of $\Delta[Ca^{2+}]_i$ through changes of emitted fluorescence intensity (F) in sequential images.
4. After cells recover homeostasis (~1 min), apply 5 μM ionomycin (Ca^{2+} ionophore) to record maximum fluorescence intensity (F_{max}).
5. After 30 s, add 30 mM EGTA to measure minimum fluorescence intensity (F_{min}) (**Note 5**).

3.2.3 Data Analysis

1. First define elliptical regions of interest (ROI) for at least 40 cells (**Note 6**).
2. Set an ROI upon a region without cells and set as background ROI.
3. Measure the intensities of all ROIs.
4. Export numeric data from Nis elements program to the Excel sheet program.
5. Normalize raw data by subtracting background emitted fluorescence intensity (F_B).
6. Calculate F (peak height following stimulation by agonist solution) and mean values of F_0, Fmax, and Fmin for each cell.
7. Ratio F/F_0 is used for defining cells responding to agonist application. Values higher than 1.5 are considered as significant increase of $\Delta[Ca^{2+}]_i$ (**Note 7**).
8. Use the following equation for calculating $[Ca^{2+}]_i$ peak values and kinetics:

$$\left[Ca^{2+}\right]_i = K_d \cdot \frac{[F - Fmin]}{[Fmax - F]}$$

9. where Fmin is the fluorescence intensity of the indicator in the absence of calcium, Fmax is the fluorescence of the

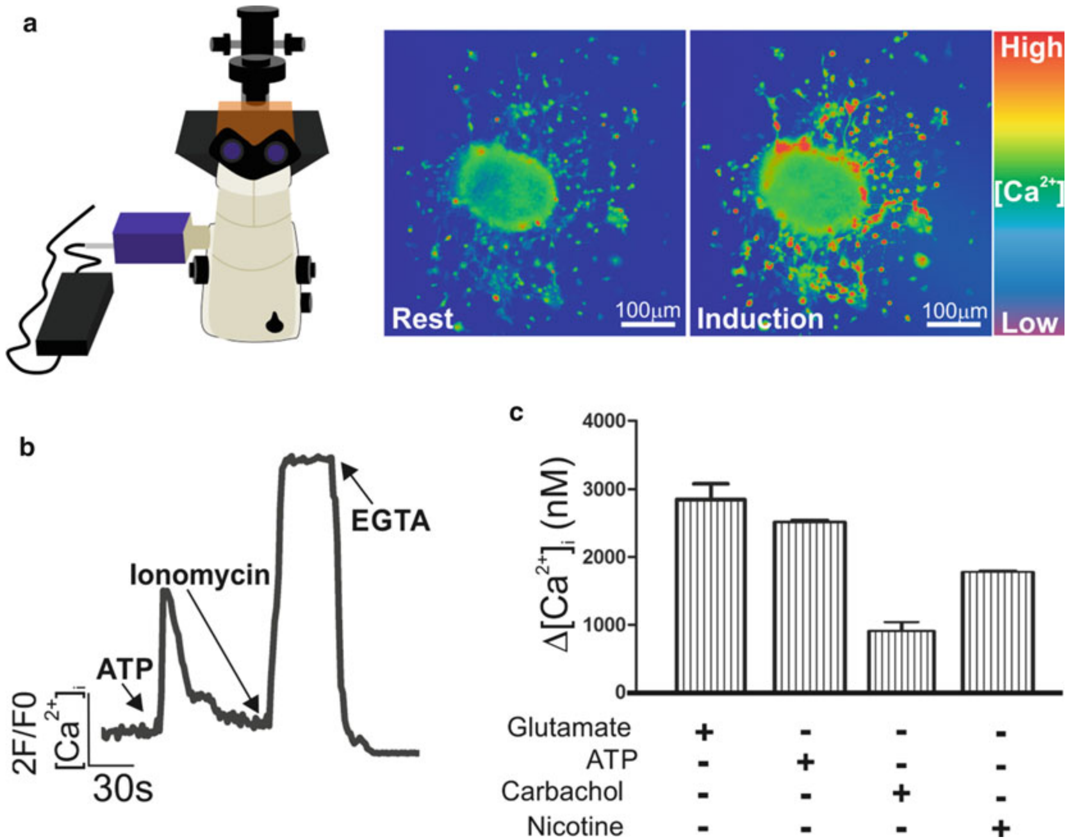

Fig. 3 Calcium imaging data plotting examples. After recording fluorescence intensities corresponding to $[Ca^{2+}]_i$, data can be usually plotted as density scale with rainbow mask (**a**), single-cellular normalized fluorescence traces (**b**), or amplitude graphs (**c**)

calcium-saturated indicator, and F is the emitted fluorescence intensity during $\Delta[Ca^{2+}]_i$. K_d is the dissociation constant for Fluo3 AM-Ca^{2+} binding, which is 325 nM (**Note 8**).

10. Another way is to plot variations in $[Ca^{2+}]_i$ as function of time relative to the basal fluorescence (F_0, $[Ca^{2+}]_i$ of resting cells) $[Ca^{2+}]$ determined from independent measurements (Fig. 3 and **Note 9**).

3.3 Calcium Microfluorimetry (FlexStation 3)

3.3.1 Calcium Indicator Incorporation

1. Remove the "FLIPR Calcium 4 Assay Dye" from the −20 °C freezer and allow equilibration for about 5 min to room temperature.
2. Add 10 ml of the Component B buffer with Probenecid to the "FLIPR Calcium 4 Assay Dye" vial and mix up and down several times until dissolved (It should look like cherry Kool-Aid).
3. Remove the cell plate from incubator. Using a multichannel pipettor, add 100 μl staining buffer to each well of the plate that already contains 100 μl of cell culture medium. Incubate for 1 h at 37 °C.

Table 2
Parameter settings for calcium measurements using FlexStation 3 (molecular devices)

Parameter	Value
Excitation wavelength (nm)	485
Emission wavelength (nm)	525
Emission cutoff	515
Sensitivity of readings	6
Sensitivity of PMT	High
Run time	60 s
Run interval	~1,52 s
Pipettor height	225 μl
Sample volume	50 μl
Dispense speed	1

4. Meanwhile, prepare the compound dilution plate with the desired agonist concentrations.
5. Make up a compound plate by applying the agonists on 96-well V-bottom plate with a multichannel pipettor (**Note 10**).
6. Set up the FlexStation equipment with the proper parameters and template info (Table 2).
7. Once the assay plate has incubated for an hour, place it in the FlexStation and click "Read."

3.3.2 Setting Parameters

Set parameters (Table 2).

3.3.3 Data Analysis

1. First in the reduction icon set kinetic reduction to Max-Min, and at baseline options choose % baseline.
2. Then in the display icon choose reduced and display number.
3. Select the plate and export to Excel spreadsheet.

4 Notes

1. E14tg2a cell line: 129/Ola-derived HPRT-negative ES cells. HPRT-minigene can be used as an additional option of selectable marker by combination with HAT selection.
2. Do no not exceed 1 % of DMSO at final concentration, or cells may die.
3. EBs are already observed 2 days after the seeding.

4. Increasing signal to noise ratio: 30 min is enough to incorporate the dye for optimal calcium live imaging. You can diminished or increase this time according to the level of background imaging brightness.
5. For better results, keep dish with the cells dish in an isolated chamber at 37 °C, during calcium imaging.
6. The ROI must fit all cells and mark brightest signal location.
7. It is noteworthy that the calcium-binding and spectroscopic properties of fluorescent indicators can vary quite markedly in cellular environments. For example, Fluo-3 fluorescence in the nucleoplasm has been found to be twice than that in the cytoplasm under conditions of normalized indicator and Ca^{2+} concentration. In addition, BAPTA-based indicators such as Fluo-3 and Fluo-4 bind various heavy metal cations.
8. For proper analysis the equipment should be calibrated using Molecular Probes' Calcium Calibration Buffer kit.
9. No calibration with known [Ca^{2+}] solutions is needed.
10. Agonist concentrations must be 5× higher than the final desired concentration.
11. Some complementary studies report directed differentiation into a single cell type, such as dopaminergic, glutamatergic, motor, serotoninergic, or GABAergic neurons [16–21].

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In Vitro Differentiation of Pluripotent Stem Cells into Functional β Islets Under 2D and 3D Culture Conditions and In Vivo Preclinical Validation of 3D Islets

Bipasha Bose and Sudheer Shenoy P

Abstract

Since the advent of pluripotent stem cells, (embryonic and induced pluripotent stem cells), applications of such pluripotent stem cells are of prime importance. Indeed, scientists are involved in studying the basic biology of pluripotent stem cells, but equal impetus is there to direct the pluripotent stem cells into multiple lineages for cell therapy applications. Scientists across the globe have been successful, to a certain extent, in obtaining cells of definitive endoderm and also pancreatic β islets by differentiating human pluripotent stem cells. Pluripotent stem cell differentiation protocols aim at mimicking in vivo embryonic development. As in vivo embryonic development is a complex process and involves interplay of multiple cytokines, the differentiation protocols also involve a stepwise use of multiple cytokines. Indeed the novel markers for pancreas organogenesis serve as the roadmaps to develop new protocols for pancreatic differentiation from pluripotent stem cells. Earliest developed protocols for pancreas differentiation involved "Nestin selection pathway," a pathway common for both neuronal and pancreatic differentiation lead to the generation of cells that were a combination of cells from neuronal lineage. Eventually with the discovery of hierarchy of β cell transcription factors like Pdx1, Pax4, and Nkx2.2, forced expression of such transcription factors proved successful in converting a pluripotent stem cell into a β cell. Protocols developed almost half a decade ago to the recent ones rather involve stepwise differentiations involving various cytokines and could generate as high as 25 % functional insulin-positive cells in vitro. Most advanced protocols for β islet differentiations from human pluripotent stem cells focused on 3D culture conditions, which reportedly produced 60–65 % functional β islet cells. Here, we describe the protocol for differentiation of human pluripotent stem cells into functional β cells under both 2D and 3D culture conditions.

Keywords: Human embryonic stem cells, Human pluripotent stem cells, β islet differentiation, Insulin, 2D differentiation, 3D differentiation

1 Introduction

As pluripotent stem cells (PSC) have the enormous potential to generate all three lineages, they are prospective candidates for biomedical research. Applications of PSC in biomedical research involve cell therapy/regenerative medicine and drug screening models. Diseases of global importance wanting regenerative medicine based cure are diabetes, stroke, Parkinson's disease, spinal cord injury, hematological malignancies, blindness deafness, osteoarthritis, kidney failure.

Hence, generation of scalable quantities of various cell types from PSC make use of various in vitro differentiation protocols will have enormous applications in regenerative medicine. Furthermore, differentiations of patient-specific PSCs have also found applications in basic research for various in vitro disease models (1–3).

1.1 Correlation Between In Vivo Development and Protocols for Differentiating Pluripotent Stem Cells into All Three Lineages, Ectoderm, Mesoderm, and Endoderm

The main goal of any in vitro differentiation protocol is to recapitulate the in vivo developmental ontogeny. Importantly, the design of in vitro cellular transitions leading to the development of ectoderm, mesoderm, or endoderm is based on the identification of intrinsic factors governing early embryonic development (4–7). These intrinsic factors are cytokines that are secreted from the different parts of the developing embryo that, in turn, influence the sequential expression of a cohort of genes (8). For example, the single layered blastula, during the early phase of embryonic development, first gives rise to the primitive streak, the body axis in mammals. The formation of primitive marks the beginning gastrulation (9). In the gastrulation stage, embryo gets organized for the first time into tri-germ lineage, ectoderm, mesoderm, and endoderm during the third week of gestation in humans. The physical events taking place during gastrulation involve the ingression of cells from the epithelial epiblast (embryonic ectoderm) through the primitive streak to give rise to the mesoderm and endoderm cell layers. The cellular events include epithelial–mesenchymal transitions (EMT) of the epiblast epithelia in which the epithelial phenotype of the epiblast cells get downregulated to give rise to a migratory mesenchymal phenotype (10). The mesoderm cells thus maintain a migratory phenotype and the endoderm cells reestablish cell–cell junctions to form a contiguous layer at the base of the primitive streak. The fibroblast growth factors (FGFs) are the important cytokines involved in mesoderm inductions and hence play a significant role in gastrulation (4). FGF receptors are involved in upregulating early mesodermal genes like brachyury and Tbx6 (4). Also, under the influence of FGF-signaling there takes place a concomitant decrease in epithelial genes like E-Cadherin and Snail (4).

The methods employed to differentiate pluripotent stem cells (PSC) into various lineages involve the first step either as the formation of three-dimensional aggregates known as embryoid bodies (EBs), or culture of PSC monolayers on various extracellular matrix proteins or culture of PSCs directly on supportive stromal layers (11). However, the first step of the majority of in vitro differentiation protocols from pluripotent stem cells (PSCs) involves the formation of 3D PSC aggregates, in the absence of anti-differentiation agents. Such 3D PSC aggregates are called embryoid bodies (EBs). EBs exhibits an interesting phenomenon of spontaneous differentiation into all three germ layers, and hence can be considered at par with the gastrula of an

embryonic development. Indeed, spontaneous differentiation of EB involves FGF signaling like gastrulation (12). The initial events in EB differentiation is characterized by the primitive endoderm fate specification of cells of EBs located at the exterior (13). Under in vivo situations during mouse development, presence of Fgfr$^{-/-}$ progenitor cells are associated with the improper development of mesodermal and endodermal cells during gastrulation (14). Importantly, homozygous null Fgfr$^{-/-}$ embryos, die in during gastrulation (15, 16). On similar lines, targeted disruption of Fgfr in EBs reportedly blocked the maturation of visceral endoderm and cavitations in mouse embryoid bodies (13).

As the primitive streak divides the embryo into the rostrocaudal axis, the mesendoderm signals of Nodal/activin and FGF families are restricted only to the caudal end (17). So the noncaudal end gives rise to ectoderm cells: the central nervous system (CNS), the cranial placodes and neural crest cells, which together form the peripheral nervous system (PNS) and the skin. Also, during the initial patterning of the ectoderm, Wnt signaling comes at the top of the regulatory cascade and inhibits BMP activity in the epidermal ectoderm and FGF activity in the neural ectoderm (18). Similarly, the approaches used for in vitro neuronal differentiation from ESC/PSC aim at either generating regionally specified neural progenitor cells and/or differentiated neuronal/glial subtypes. Some of these protocols go via EB formation, similar to endodermal differentiation protocols while some of them are direct differentiation protocols. EB formation is carried out in the presence of retinoic acid (RA) (19). Also, RA is a developmentally regulated morphogen and has been reported to induce primary neurons in Xenopus (20, 21) hindbrain specification (22) and also motor neuron specification (23). Furthermore, during PSC differentiation into neuronal lineage, interplay of other cytokines and RA, which in turn, maintains a sustained activity of RA are most crucial events for the generation of neurons (24). As Wnt is the first cytokine responsible for neuronal development, activation of Wnt via overexpression of β catenin or treatment of the ES cells with Wnt 3a conditioned media resulted in the generation of neurons (25). Most of the neuronal differentiation protocols from human PSC require the supplements like b-FGF and Noggin (26). Noggin is responsible for the inhibition of BMP signaling, a prerequisite for neurogenesis (18, 27). Also, similar to the post-gastrulation events, the neuroepithelial cells in differentiation protocols are directed to form various neural cell types (27).

In PSC differentiation protocols, development of the primitive streak (PS) like population of gastrula and mesoderm induction is monitored by the expression of Brachyury (T). Cytokines like BMP4, when added to the EBs or directly to the PSCs, indirect differentiation protocols, generates Brachyury positive PS like cells

followed by $FLk1^+$ mesodermal progenitors (28–31). On the other hand, if Wnt signaling responsible for mesodermal patterning, when blocked in the differentiation protocols, resulted in the loss of T^+ PS-like cells and mesodermal progenitors. Thus, loss of T^+ cells in the in vitro PSC differentiation protocols upon blocking of Wnt signaling post PS development correlates with the active role of Wnt signaling in PS and mesoderm development (32, 33). Alternatively, addition of Wnt in the PS-like induced PSC induced cardiac mesoderm formation (34).

It is activin/Nodal signaling that plays a significant role in the development of definitive endoderm. Accordingly, all the endodermal in vitro differentiation protocols from PSC use Activin A for the induction of definitive endoderm (35–40). However, previous to DE, activin treatment in PS-like cells obtained from PSC results in the formation of mesoderm and endoderm (mesendoderm) as evident by the co-expression of Brachyury, FoxA2, and Gossecoid (41, 42). Interestingly, the GSC positive cells are capable of giving rise to mesoderm and endoderm, and hence are called mesendoderm progenitors. Furthermore, the DE cells can be induced into hepatic and pancreatic lineage in a stepwise fashion using a cocktail of cytokines.

1.2 Evolution of Protocols for Insulin-Producing β Islets from PSC and Their Respective Strategies and Success

Mimicking in vivo pancreas development in PSC differentiation protocols and obtaining scalable quantities of β islets can be quite complex. This complexity of differentiation protocols is attributed to the complexity of an adult pancreas, which comprises of exocrine (produce digestive enzymes) and endocrine (α, β, and pancreatic polypeptide producing cells) (43). Earliest attempts to obtain functional pancreatic β islets did not mimic the chronological in vivo events in pancreas organogenesis. Rather, the protocol involved a straightforward "Nestin selection pathway," which made use of neuronal cues (44). The underlying reasons for using the "Nestin selection pathway" was due to the fact that pancreatic endoderm and neural ectoderm co-express a large number of markers suggesting a probable common pathway or cross talk between neuronal and pancreatic differentiation. For example, inhibitory sonic hedgehog from notochord promotes pancreatic organogenesis (45), innervations of β islets by neurons (46), Schwann cells surrounding β islets (47), and influence of such neural crest-derived neurons and Schwann cells on proliferation and maturation of β islets (48). Later on, this protocol exhibited serious flaws and was rejected because the insulin-positive cells were not because of de novo insulin synthesis, rather because of the uptake of insulin from the culture medium (49). Recently, Arntfield et al. (50) reported the presence of developmentally distinct progenitors of neural crest and pancreatic origin, in an adult mammalian pancreas.

Second generation of β islet differentiation protocols involved the overexpression of β transcription factors. After the discovery of transcriptional hierarchy of factors responsible for β cell organogenesis, scientists tried to perform forced expression of such factors. β cell transcription factors like Pdx1, Pax4 and Nkx2.2, upon overexpression in ESC resulted in the formation of β cells (51–53). Such protocols involved transfection methods and produced low percentage ~1 % insulin-positive cells (53).

Third generation of β islet differentiation protocols from PSC adopted an approach thereby mimicking the stepwise pancreatic organogenesis. The various steps involved in such protocols were definitive endoderm followed by sequentially priming the cells into primitive gut tube, posterior foregut, pancreatic endoderm, and endocrine precursors. Various protocols using this stepwise pancreas organogenesis approach were published by several research groups worldwide. (38–40, 54, 55, 57–59). However, all these protocols either involved a 2D/monolayer or a 3D approach to obtain β islets. In 3D protocols, 3D approach was used to obtain the functional β islets in the last step of differentiation, and proved to be more efficient as compared to the 2D protocols. The differences amongst all these protocols were the use of cytokines in various steps in the differentiation. The first step in definitive endoderm (DE) differentiation from PSC for most of these protocols involved the use of Activin A/a TGFβ family member, alone or in combination of other molecules. The choice of Activin A to induce DE was based on the studies of early embryonic development in different model systems that emphasized the role of nodal, a soluble molecule of TGFβ/Activin signaling family during gastrulation. Nodal promoted DE and mesoderm and a higher level of nodal was reported to facilitate endoderm specification in these model systems (60–63). D'Amour et al. (37, 54) used a combination of Activin A and Wnt3a; Jiang et al. (57) used Activin A and sodium butyrate in the complete absence of serum; Philips et al. (64), however, used BMP4 along with Activin A; Shi et al. (55) used Activin A and Retinoic acid; and Zhang et al. (65) had several other components like 0.2 % BSA, N2, B27, along with Activin A to induce DE. Bose et al. (40) used a combination Activin A and Retinoic acid in the first step for to obtaining DE cells. However, one protocol did not use Activin A for DE differentiation from hESC. Rather, this group used the old protocol of Nestin selection, in combination with Exendin-4, a GLP-1 analog for maturation of pancreatic progenitors (66). Interestingly, the protocol of Mao et al. (66) did not produce DE cells and the cells were directly enriched for pancreatic progenitors. Also, more recently, Rezania et al. (59) have not used Activin A for the induction of definitive endoderm. Instead, GDF8 (a TGFβ family member) was used in combination of GSK3β inhibition, in accordance with the concept of Naujok et al. (67). As per Naujok et al. (67), early steps of DE formation from hPSC require high levels of canonical Wnt signaling, GSK3β inhibition and low levels of Activin A.

In the next step to obtain primitive gut tube or posterior foregut, the growth factors used were a combination of cyclopamine (sonic hedgehog/shh inhibitor), noggin, b-FGF, and retinoic acid in the most of the aforementioned differentiation protocols that mimicked pancreatic organogenesis. The decision to use cyclopamine, noggin, b-FGF and retinoic acid to induce posterior foregut was based on reports from developmental biology and successes of various differentiation protocols from PSC that emphasized the role of such molecules in pancreas organogenesis (68–71).

Finally, for obtaining functional pancreatic endocrine progenitors and β cells, most of the protocols have used Exendin-4 in the culture media to stimulate insulin secretion, β cell proliferation (37, 54, 55, 64, 65) alone or in combination with betacellulin and Hepatocyte growth factor (HGF). The decision to use exendin-4, a GLP-1 analog was made based on previous reports about the role GLP-1 to promote insulin secretion from β cells (72) betacellulin to promote β cell proliferation (73–75) and HGF also to promote β cell growth and increase insulin production by β cells (76, 77).

Amongst all the third-generation 2D β islet differentiation protocols, different percentages of functional insulin-producing cells were obtained. The earlier protocols by Shi et al. (55) and D'Amour et al. (37) showed limited glucose sensitivity but showed sensitivities to a variety of secretagogues. β islets obtained in pancreatic differentiation protocol by Kroon et al. (38) exhibited high levels of in vivo glucose responsiveness. More recent pancreatic differentiation protocol of Nostro et al. (39) reported a generation of 25 % insulin-positive cells. 3D differentiation protocols evolved after the 2D differentiation protocols and were essentially able to mimic the in vivo conditions and strengthen the cellular interactions probably by improved secretory dynamics and electrical coupling of insulin-producing cells (78). Also, β cell proliferation and functions reportedly increase due to cell–cell interactions and signaling with extracellular matrices as available under 3D differentiation conditions (79–81). Indeed, the 3D pancreatic differentiations from mouse ESC/iPSC and human PSC gave rise to a high percentage of functional insulin-producing β cells. 60 % insulin producing cells were reported in case of pancreatic differentiations from mouse ES, iPSC and mouse fetal pancreas (82, 83). 65 and 50 % insulin producing cells were, however, reported in case of pancreatic differentiations from hESC (40) and (Rezania et al., 2014) (59) respectively. Interestingly, the differentiation protocol by Rezania et al. (2014) (59) had adopted a 3D approach right from the early stage of pancreatic endoderm.

1.3 Details of Our Protocol for In Vitro PSC Differentiation into β Islets

Our method of β islet differentiation from hESC also followed the sequential in vivo pancreatic organogenesis like all other third generation β islet differentiation protocols. This method is a 42-day protocol, a combination of monolayer 2D followed by a 3D differentiation. All the initial steps from DE induction till the generation of β islets were carried out under 2D monolayer conditions until 32 days (Fig. 1). Last 10 days of the protocol involved the culturing of 2D β islets under 3D conditions for β cell maturation. The first 32 days of 2D differentiation resulted in the formation of ~24.5 % functional β islets (Bose et al. (40)). However, upon 10 days of further maturation of these 2D β islets under 3D conditions, ~65 % of functional β islets were obtained (Bose et al. (40)). Amongst the first 32 days of differentiation, a combination of 2D and 3D approach was taken where spontaneously differentiated EBs, definitive endoderm, pancreatic endoderm and β islets were obtained. Spontaneous tri-lineage differentiation was first carried out in 3D by making of embryoid bodies (EBs) from hESC for first 48 h (**step 1**, Figs. 1 and 2). EBs were then plated onto Matrigel and subjected to sequential treatment of cytokines to induce the DE, pancreatic endoderm and finally the pancreatic endocrine/β cells (**steps 2–4**, Figs. 1 and 2). EBs plated onto Matrigel and treated with Activin A and retinoic acid for 6 days generated DE cells, which were characterized by the presence of Sox 17, CXCR4 and Fox A2 as DE marker and loss of Oct3/4 as a proof of concomitant loss of pluripotency (**step 2**, Figs. 1, 2, and 3). The DE cells were then induced with Noggin and b-FGF to form pancreatic endoderm (PE) for 12 days (**step 3**, Fig. 1). The successful conversion of cells into PE was evident from the expression of PDX1 (the first transcription factor in pancreatic organogenesis) along with NGN3, NKX6.1, and flattened morphology of cells (**step 3**, Figs. 2 and 3). The presence of E-Cadherin is the hallmark of a cell with an epithelial behavior (Fig. 2).The PE cells were then induced with b-FGF, nicotinamide and GLP-1 for pancreatic

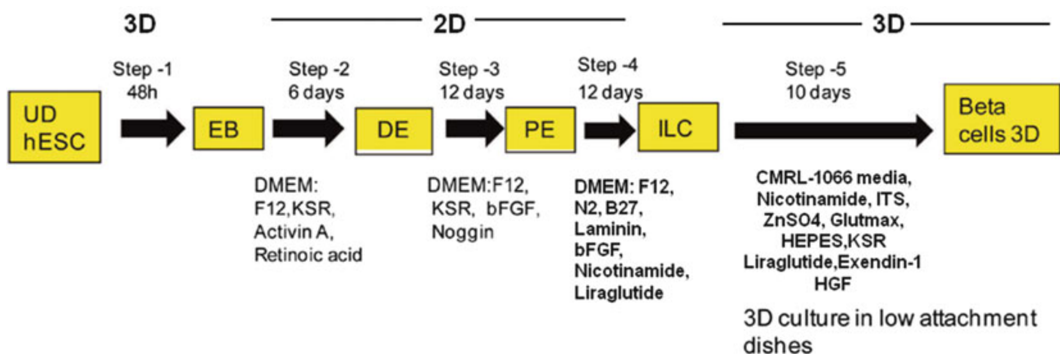

Fig. 1 Scheme of pancreatic differentiation protocol (Bose et al. 2012 (40), Reproduced with permission from Wiley)

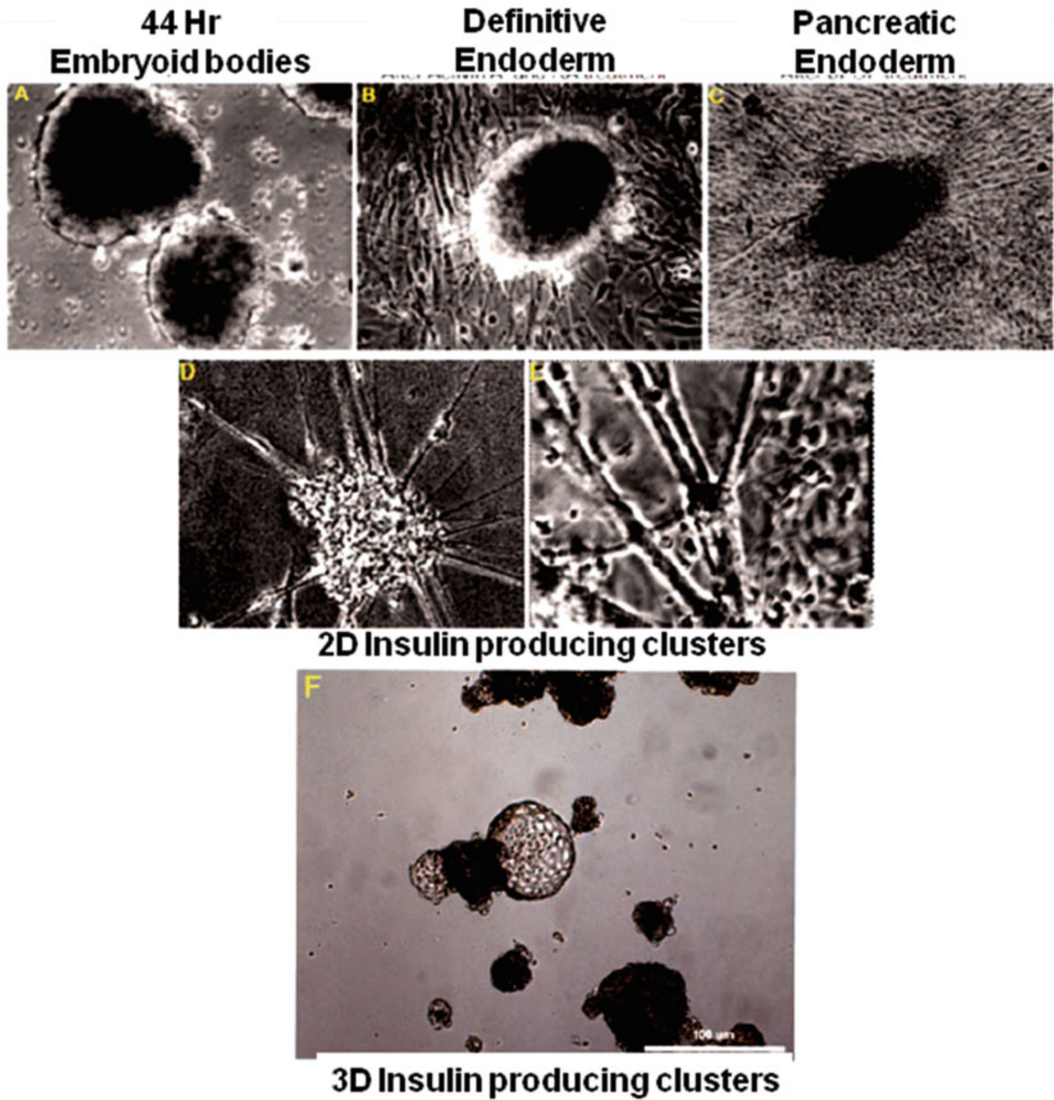

Fig. 2 Morphologies acquired by the cells during sequential differentiation process (Bose et al. 2012 (40). Supplementary information. Reproduced with permission from Wiley)

endocrine/β cell development, formation of islet-like clusters (ILC) in 2D (**step 4**, Figs. 1 and 2). The co-localization of insulin and c-peptide confirmed the presence of ILC in **step 4** (Fig. 3). Further maturation of pancreatic endocrine cells was done under 3D conditions using CMRL media, which is protein free, vitamins and nucleoside rich media, used otherwise, for maintaining human β islets in culture. Liraglutide, a long-lived GLP-1 agonist also complemented for the maturation of β islets in 3D as evident from presence of mature β cell markers like MafA and exendin-1, a vasoactive peptide stimulating the release of insulin from the mature β cells (**step 5**, Figs. 1, 2, and 3).

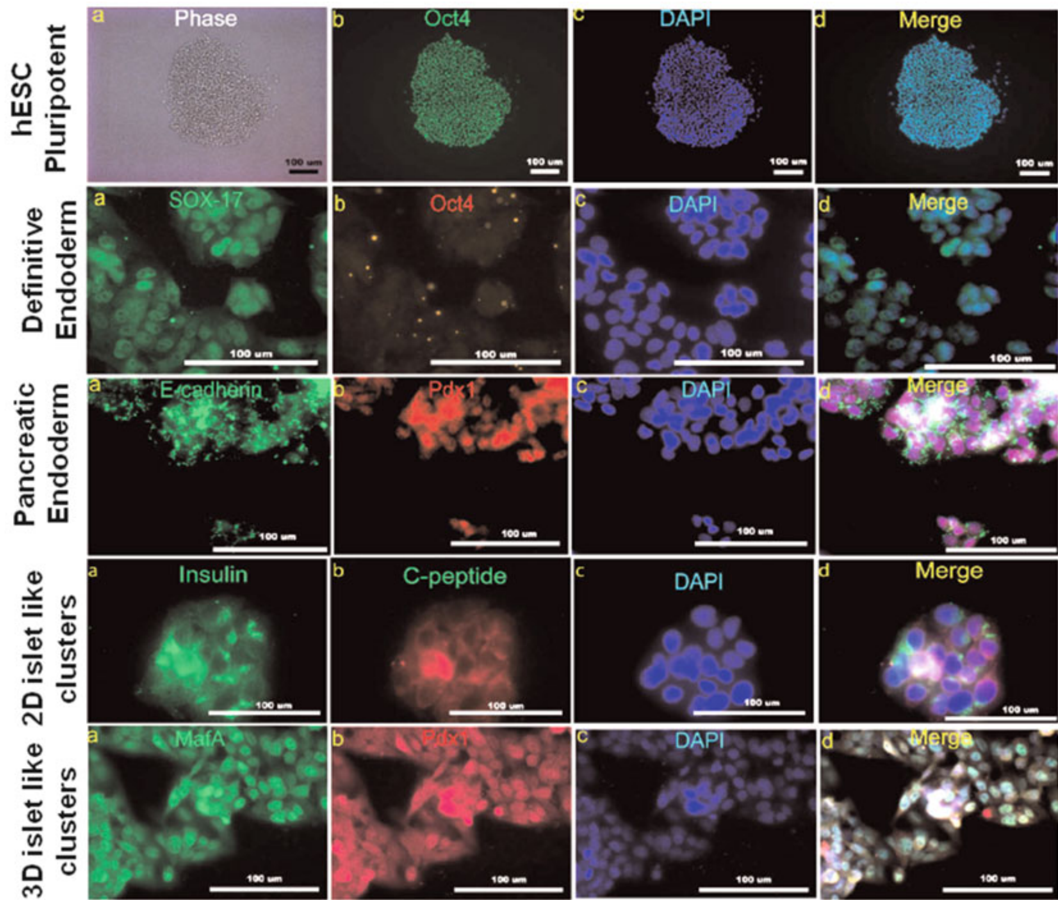

Fig. 3 Expression of markers by immunofluorescence during steps 1–5 of the differentiation (Bose et al. 2012 (40), Reproduced with permission from Wiley)

2 Materials

2.1 Cell Lines, Media, and Supplements

1. ReliCell hES1 (Human embryonic stem cell lines ReliCell hES1 (Mandal et al. 2006), BGO1 (ATCC)) (84), BGO1 (ATCC), Mouse Embryonic Fibroblast (Mandal et al.) (84).
2. Culture Dish (35 mm, Nunc 153066).
3. Ultra-Low Attachment culture dishes (60 mm, Corning, CLS3261).
4. Gelatin (Sigma G1393).
5. PBS.
6. 0.05 % Trypsin–EDTA (GIBCO, Life Technologies, 25300-054).
7. Matrigel (BD Biosciences, San Jose, CA, 354230).
8. Mouse embryonic fibroblast medium (Store at 4 °C).

DMEM-high glucose (GIBCO, Life Technologies, 11995-065)	500 mL
FBS (Hyclone, SH30071.03, Logan, UT)	50 mL
L-Glutamine (GIBCO, Life Technologies, 25030-081)	5 mL
MEM-Non Essential Amino acids (GIBCO, Life Technologies, 11140-050)	5 mL
0.1 mM 2-mercaptoethanol (GIBCO, Life Technologies, 21985-023)	500 μL

9. Human embryonic stem cell medium (Store at 4 °C).

DMEM/F-12 (GIBCO, Life Technologies, 11320-033)	500 mL
FBS (Hyclone, SH30071.03, Logan, UT)	75 mL
Knockout serum replacement (GIBCO, Life Technologies, 10828-028)	25 mL
L-Glutamine (GIBCO, Life Technologies, 25030-081)	5 mL
MEM-non essential amino acids (GIBCO, Life Technologies, 11140-050)	5 mL
0.1 mM 2-mercaptoethanol (GIBCO, Life Technologies, 21985-023)	500 μL

10. Supplements for Human embryonic stem cells.

    bFGF (R&D Systems, 233-FB-025): Stock solution at 25 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 embryonic stem cell medium at a final concentration of 4 ng/mL.
11. Differentiation Medium for Human embryonic stem cells.
    - Embryoid body formation media and Basal media for definitive endoderm differentiation (store at 4 °C)

DMEM/F-12 (GIBCO, Life Technologies, 11320-033)	500 mL
Knockout serum replacement (GIBCO, Life Technologies, 10828-028)	50 mL
L-Glutamine (GIBCO, Life Technologies, 25030-081)	5 mL
MEM-non essential amino acids (GIBCO, Life Technologies, 11140-050)	5 mL
50 mM 2-mercaptoethanol (GIBCO, Life Technologies, 21985-023)	500 μL

12. Supplements for definitive endoderm differentiation medium.

    Activin A (Sigma, A4941): Prepare a stock solution at 50 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 definitive endoderm differentiation basal medium at a final concentration of 100 ng/mL.

    All trans-Retinoic Acid (Sigma, R2625): Prepare a stock solution at 10 mM in DMSO (Sigma, D2650). Aliquot into 100 μL and store at −80 °C. Once thawed, keep at 4 °C with protection from light. Add to human definitive endoderm differentiation basal medium to make a final concentration of 1 mM.

13. Human pancreatic endoderm specification basal media (store at 4 °C).

DMEM/F-12 (GIBCO, Life Technologies, 11320-033)	500 mL
Knockout serum replacement (GIBCO, Life Technologies, 10828-028)	50 mL
L-Glutamine (GIBCO, Life Technologies, 25030-081)	5 mL
MEM-non essential amino acids (GIBCO, Life Technologies, 11140-050)	5 mL
50 mM 2-mercaptoethanol (GIBCO, Life Technologies, 21985-023)	500 μL

14. Supplements for pancreatic endoderm specification medium.

    bFGF (R&D Systems, 233-FB-025): Stock solution at 25 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 pancreatic endoderm specification medium at a final concentration of 20 ng/mL.

    Noggin (Sigma, H6416): Stock solution at 25 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 pancreatic endoderm specification medium at a final concentration of 100 ng/mL.

15. Media for the generation of Islet like clusters (store at 4 °C).

    DMEM/F-12 (GIBCO, Life Technologies, 11320-033) 500 mL.

16. Supplements for the media for the generation of Islet like cluster.

    1 % N2 supplement (GIBCO, Life Technologies, 17502-048), 2 % B27 supplement (GIBCO, Life Technologies, 10889-038) added to the media accordingly.

    bFGF (R&D Systems, 233-FB-025): Stock solution at 25 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 pancreatic endoderm specification basal medium to a final concentration of 20 ng/mL.

Nicotinamide (Sigma, N0636): Stock solution at 1 M in PBS. Aliquot into 5 mL and store at −20 °C. Once thawed, keep at 4 °C. Add to human pancreatic endoderm specification basal medium to a final concentration of 10 mM.

Liraglutide (Novo Nordisk): Prepare a stock solution at 10 mM in PBS. Aliquot into 100 μL and store at −80 °C. Once thawed, keep at 4 °C with protection from light. Add to human pancreatic endoderm specification basal medium to a final concentration of 10 nM.

17. Media for the generation of 3D Islet like clusters (store at 4 °C).

CMRL Medium-1066 (GIBCO, Life Technologies, 11530-037)	500 mL
Knockout serum replacement (GIBCO, Life Technologies, 10828-028)	50 mL
Glutamax (GIBCO, Life Technologies, 35050-061)	5 mL
HEPES (GIBCO, Life Technologies, 15630-080)	5 mL

Nicotinamide (Sigma, N0636): Stock solution at 1 M in PBS. Aliquot into 5 mL and store at −20 °C. Once thawed, keep at 4 °C. Add to 3D Islet like clusters generation basal medium to a final concentration of 10 mM.

Zinc Sulfate Monohydrate (Sigma, 96495): Stock solution of 0.1 M in PBS. Filter sterilize and aliquot into 1 mL and store at −20 °C. Add to the 3D islet cluster generation basal media to a final concentration of 0.1 mM.

Exendin-1 Peptide (Abbiotec, 350215): Stock solution of 1 mg/mL (~0.2 mM) in sterile PBS. Aliquot 100 μL and store at −20 °C. Add to the 3D islet cluster generation basal media to a final concentration of 20 nM.

Liraglutide (Novo Nordisk): Prepare a stock solution at 10 mM in PBS. Aliquot into 100 μL and store at −80 °C. Once thawed, keep at 4 °C with protection from light. Add to 3D Islet like clusters generation basal medium to a final concentration of 10 nM.

HGF (R&D Systems 294-HGN): Stock solution at 10 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 3D Islet like clusters generation basal medium to a final concentration of 20 ng/mL.

2.2 Gene Expression Analysis by qRT-PCR

1. RNeasy Mini Kit (Qiagen, Cat. No 74104 Germany).
2. Random hexamers and Superscript II (Invitrogen, Life Technologies Cat. No: 11904-018).

Table 1
List of primers used and their respective sequences

Name of the gene	Sequence	T_m (°C)	Accession no.
Oct4	CGGAAGCTGGAGAAGGAGAAGCTG(F) CAAGGGCCGCAGCTTACACATGTTC(R)	58	NM_002701.4
Foxa2	AGAAGCAACTGGCACTGAAGGA(F) GTAGTGCATGACCTGTTCGTAG(R)	55	NM_021784.4
Sox-17	AGCAGAATCCAGACCTGCAC(F) TTGTAGTTGGGGTGGTCCTG(R)	60	NM_022454.3
Glut-2	TGACATGAACAGAGAAACAATAAGGG(F) ATGACATTTCTGATGAGAGCACA(R)	52	NM_000340.1
Pdx-1	CCCATGGATGAAGTCTACC(F) GTCCTCCTCCTTTTCCAC(R)	51	NM_000209.3
Pancreatic polypeptide	CTCTGTTACTACAGCCACTG(F) AGTCGTAGGAGACAGAAGGT(R)	52	NM_002722.3
Insulin	GCCTTTGTGAACCAACACCTG(F) GTTGCAGTAGTTCCAGCTG(R)	54	NM_000207.2
Ngn3	CTCGAGGGTAGAAAGGATGACGCCTC(F) ACGCGTGAATGGGATTATGGGGTGGTG(R)	63	NM_020999.3
Glucagon	CATTCACAGGGCACATTCAC(F) CGGCCAAGTTCTTCAACAAT(R)	60	NM_002054.3
Somatostatin	CCAACCAGACGGAGAATGAT(F) CCATAGCCGGGTTTGAGTTA(R)	60	NM_001053.3
MafA	TCAACGACTTCGACCTGATG(F) CGCTCATCCAGTACAGATCCT(R)	60	NM_201589.3
MafB	ACTGGATGGCGAGCAACTAC(F) GCTTGGTGATGATGGTGATG(R)	61	NM_005461.3
Nkx 6.1	CCTGTACCCCTCATCAAGGA(F) CTCTGTCATCCCCAACGAAT(R)	60	NM_145285.2
GAPDH	CCTGAACCCTAAGGCCAACCGTGAA(F) ATACCCAAGGAAGGCTGGAAAA(R)	66	NM_001001303

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3. SYBR Green Platinum SYBR Green qPCR Supermix-UDG (Invitrogen, Life Technologies Cat. No: 11733-046).
4. CFX-96/Real Time system C1000-Thermal cycler (Bio-Rad).
5. Primers used at a stock concentration of, 100 μm (Sigma), Primers (Table 1) reconstituted in DNase-RNase free water (Ambion, Life Technologies, AM-9937) at 5 μm and stored at −20 °C freezer.

Table 2
List of primary antibodies and their respective dilutions used for immunocytochemistry and flow cytometric analysis

Antibody	Dilution	Company	Catalog number
Oct4	1:100	Santa Cruz	SC-9081
MafA	1:100	Santa Cruz	SC-27140
Sox17	1:100	R&D Systems	AF-1924
CXCR4	1:100	Abcam	AB-2074
E-cadherin	1:100	Millipore	SC-8426
Foxa2	1:100	Santa Cruz	SC-6554
Insulin	1:20	Santa Cruz	SC-8033
Pdx-1	1:100	Santa Cruz	SC-14664
C-peptide	1:100	Millipore	05-1109
Somatostatin	1:100	Santa Cruz	SC-7819
Glucagon	1:100	Santa Cruz	SC-13091

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2.3 Immunofluorescence Staining of Cultured Cells

1. Eight-well chamber Permanox slides (Thermo Scientific 177445).
2. Fixation Buffer: 4 % (w/v) paraformaldehyde (PFA) (Sigma P6148).
3. Washing buffer: PBS with 100 mM glycine (Sigma G8898), 0.3 % (v/v).
4. Triton X-100 (Sigma T9284).
5. Blocking buffer: PBS with 5 % Normal donkey serum (Sigma D9663).
6. Primary antibodies with recommended dilution have given in Table 2.
7. Secondary antibodies with recommended dilution have given in Table 3.
8. Mounting media with DAPI (Sigma F6057).

2.4 Flow Cytometry

1. FC Fixation Buffer: 4 % (v/v) PFA in PBS.
2. FACS Buffer: PBS with 2 % (w/v) fetal bovine serum (Hyclone, SH30071.03, Logan, UT).
3. Triton X-100 (Sigma T9284) 0.1 % (v/v).
4. Primary antibodies with recommended dilution have given in Table 2. Secondary antibodies (Invitrogen) with recommended dilution have given in Table 3.

Table 3
List of secondary antibodies and their dilutions used

Antibody	Company	Catalog no.	Dilution
Donkey anti-mouse Alexa Flour 488	Invitrogen	A21202	1:1,000
Donkey anti-goat Alexa Flour 488	Invitrogen	A11055	1:1,000
Donkey anti-rabbit Alexa Flour 594	Invitrogen	A21207	1:1,000
Donkey anti-rabbit Alexa Flour 647	Invitrogen	A31573	1:1,000
DAPI	Invitrogen	D1306	1:5,000

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2.5 In Vitro Functional Assay (Insulin and C-Peptide Assay)

1. Low attachment 6-well dishes (Corning Cat. No 3471).
2. DMEM (Glucose free) (Invitrogen, Life Technologies: 11966-025).
3. Kreb's ringer bicarbonate buffer (130 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 20 mM Hepes, and 0.1 % BSA (w/v)).
4. Glucose (Sigma G8270).
5. Tolbutamide (Sigma T0981).
6. Nifedipine (Sigma N7634).
7. Diazoxide (Sigma 9035).
8. Human insulin ELISA kit and C-peptide ELISA kit (Mercodia, Sweden Catalog # 10-1172-01).

2.6 Immuno histochemistry of Tissues

1. Fixation Buffer: 4 % (v/v) PFA in PBS for fixing cells.
2. 10 % Neutral Buffered formalin for fixation of the tissues.
3. Paraffin block for embedding tissues.
4. Microtome (Leica, RM2245).
5. Ethanol Grades (100, 95, 80, and 50 %, respectively).
6. Citrate buffer (Sigma W302600) for antigen retrieval.
7. Blocking buffer: 1 % BSA (Sigma) in PBS.
8. Permeabilization Buffer: 0.2 % Triton X-100.
9. Primary antibody and secondary antibodies given in Tables 2 and 3.
10. Mounting media with DAPI (Sigma F6057).
11. Fluorescence microscope (Zeiss).

2.7 In Vivo Preclinical Validation of In Vitro Derived β Islets

1. NOD-SCID mice (Jackson Labs).
2. Streptozotocin (Sigma S0130-100MG).
3. Surgical instruments.
4. Permanent glue.

5. Antibiotic ointment.
6. Anesthesia: Avertin (1:10 solution of Tribromoethanol in tertiary amyl alcohol).
7. Electrical shaver.
8. 1 mL syringe with 25G needle (BD 305903).
9. Portable Glucometer (Lifescan Inc.).

3 Methods

3.1 Derivation of Mouse Embryonic Fibroblasts (MEFs)

1. MEFs were derived from 13.5 days post-coitum fetuses at stages 21 and 22 (Theiler et al.) (85) of inbred C57BL6J mice.
2. MEFs were cultured in MEF media till confluency in 5 % CO_2 and 3 % oxygen (please refer to Section 4.2, point 6). Then, they were cryopreserved in 10 % DMSO at a cell density of 5×10^6 cells/mL until further use. Before using as feeders MEFs were mitomycin-C (10 μg/mL) treated to arrest the cell division. Mitomycin-C treated fibroblasts were prepared in large batches and stored in bulk at a concentration of 2×10^6 cells/mL.
3. The inactivated cells were then plated on 0.2 % gelatin-coated 35 mm culture dishes in MEF media at a concentration of 0.2×10^6/dish. These MEF dishes used for derivation and maintenance of hESCs.

3.2 Cell Growth and Differentiation of Human Embryonic Stem Cells

Differentiation of hESC into functional β islets was carried out as per the differentiation scheme given in Fig. 1

1. hESC undifferentiated colonies mechanically passaged on the MEF feeder cells, and the cultures were grown at 37 °C and 5 % CO_2 (please refer to Section 4.2, points 1–3 and Section 4.3, point 1 for ensuring health of the undifferentiated cells and Section 4.2 point 4, and Section 4.3, point 2 to ensure a high propensity of endodermal differentiation by hPSC).
2. Step 1: Embryoid bodies (EB) were formed by manually passaging the hESC colonies (100–150 cells per clump). These clumps were seeded on to low adherent 60 mm dishes in serum-free EB media for 48 h under normoxic conditions at 5 % CO_2 (please refer to Section 4.2, point 7 and Section 4.3, point 3 for ensuring optimal conditions for differentiation).
3. Step 2: At the end of 48 h, EBs were collected and plated in 35 mm matrigel coated culture dishes in EB medium and were allowed to adhere overnight. Simultaneously, EBS was also plated in matrigel coated 8-well Permanox slides for immunofluorescent staining at different steps of differentiation (refer to

Section 4.1 for troubleshooting issues at this step). EBs were then given fresh media change with human definitive endoderm differentiation media containing KSR: DMEM with 100 ng/mL activin A and 1 mM all *trans*-RA and were allowed to grow in this media for another 6 days with media change given every alternate day. At the end of this step, definitive endoderm cells were obtained. A portion of the cells was taken for characterization by qRT-PCR Markers like CXCR4, SOX17, FOXA2 are expressed by these cells. Also, one of the Permanox chamber slides were fixed and stained for the aforementioned DE markers (refer to Section 4.1 for troubleshooting issues to obtain a high percentage of DE cells at this step).

4. Step 3: Definitive endoderm cells obtained in the previous step were fed with pancreatic endoderm media containing KSR: DMEM/F12 with 20 ng/mL of bFGF and 100 ng/mL Noggin for another 12 days with media change given every alternate day to obtain pancreatic endoderm. A portion of the cells were taken for qRT-PCR characterization, markers like PDX1, NGN3, NKX6.1 seen expressed by these cells. One Permanox slide was fixed and assessed for the markers mentioned above by immunocytochemistry (please refer to Section 4.2, point 8 to ensure a high percentage of pancreatic endoderm).

5. Step 4: To obtain insulin-producing precursors/islet-like clusters in culture, ILC (2D), the cells obtained in previous step, were further cultured in DMEM: F12, with N2 (1 %) and B27 (2 %) supplements, 1 mg/mL laminin, 20 ng/mL bFGF, and 10 mM nicotinamide, 10 nM liraglutide for 12 days with media change given on alternate days (please refer to Section 4.1 to troubleshoot issues related to the optimal morphologies of 2D ILC). One 35 mm plate from the 2D culture was harvested by trypsinization, a portion of the cells were taken for qRT-PCR characterization for markers like Pdx1 insulin, c-peptide, Glut 2 and flow-cytometric analysis for the estimation of insulin, C-peptide, and Pdx1. Also, one of the chamber slides from **step 4** of differentiation was fixed with 4 % PFA and subjected to immunofluorescence staining for insulin and c-peptide.

6. Step 5: The islet-like clusters (ILC) obtained in 2D culture, were carefully picked up under a stereomicroscope and transferred to low attachment tissue culture dishes for culturing under them under 3D conditions. The 3D cultures of ILC were supplemented with amino acid rich media, CMRL media 1066, supplemented with KSR, 10 mM nicotinamide, ITS (insulin transferring selenium), 0.1 mM zinc sulfate, glutmax, Hepes, 10 nM Liraglutide, 20 nM Exendin-1 and 20 ng/mL HGF (hepatocyte growth factor). The islets were cultured in 3D for another 10 days with media change given on alternate days before they were harvested for molecular characterization, in vitro functional assays and

animal transplantation. For immunofluorescence analysis, the 3D ILC were plated onto Matrigel coated Permanox chamber slides in 3D islet media, allowed to adhere overnight, fixed and stained for the mature β cell markers.

3.3 Quantitative RT-PCR

1. Undifferentiated cells and cells from all stages of differentiation were trypsinized; washed twice in PBS and the cell pellets were initially stored in RLT buffer provided in the RNeasy mini kit at −80 °C so that RNA can be isolated at the same time from the samples of different steps of differentiation.
2. Total RNA extraction was done using RNeasy mini kit as per manufacturer's instructions. RNA (1 μg) was reverse transcribed into cDNA-specific transcripts using random hexamers and superscript II (Invitrogen) as per manufacturer's instructions. Real-time PCR was performed with SYBR Green: Platinum SYBR green qPCR Supermix-UDG in Bio-Rad CFX 96 C 1000 Real time PCR equipment. Relative gene expression was calculated by the δCt method compared with undifferentiated cells (day 0), using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as housekeeping gene. Primer sequences are summarized in Table S1. Gene expression analysis was done for undifferentiated hESC and hESCs differentiated into pancreatic islet-like clusters in all the five steps of differentiations. RNA sample from human fetal pancreas was used as positive control (Bose et al. (40), Supplementary data).
3. qRT-PCR reactions were run in triplicates for quantitative analysis for each experimental gene and sample along with the reference gene GAPDH using the Platinum SYBR green qPCR Supermix-UDG. 5 μL of SYBR Green qPCR Master Mix, 0.5 μL each of forward and reverse primers for each gene, 2 μL of cDNA and 2 μL water was mixed to obtain 10 μL of reaction volume for each reaction on thermal cycler.
4. Gene specific primers and their amplicon sizes are listed in Table 1. The annealing temperature for all reactions is 60 °C, and 40 cycles are sufficient to detect signal. The thermal cycler is used to monitor fluorescence throughout the reaction and conduct melt curve analysis of all samples to verify specificity.
5. Thermal cycler program is listed below.
   (a) Hot start incubation—15 min at 95 °C. (b) Denaturation—15 s at 94 °C. (c) Annealing—30 s at 60 °C. (d) Extension and data acquisition—30 s at 72 °C. (e) Repeat steps (b–d) 39 times to complete reaction.

   (b) The Thermal Cycler software generates cycle threshold (Ct) values for all samples; Relative gene expression (to GAPDH) is expressed as δCt. δCt $\geq$ 16 was considered to be the non-expression of a gene. Lower δCt corresponded to a high expression of a gene.

3.4 Immunofluorescence Staining

1. Culture medium from the chamber slides was aspirated and the cells were washed with 1× PBS carefully, so as to avoid cell detachment.
2. Cells were fixed for 10 min at room temperature with 4 % paraformaldehyde (PFA). 4 % PFA was aspirated and the cells were washed twice with PBS and then incubated with permeabilization buffer for 10 min at room temperature.
3. Cells were then blocked using blocking buffer for 1 h at room temperature.
4. Primary antibodies were prepared in fresh blocking buffer at appropriate dilution (*see* Section 2) and were incubated overnight at 4 °C.
5. Cells were washed thrice with PBS for 5 min each. Appropriate secondary antibodies of dilutions of 1:1,000 in PBS were used and the cells were incubated for 60 min at room temperature.
6. Post secondary antibody washes were carried out thrice with PBS for 5 min each. Cells were then mounted using immunofluorescence mounting medium with DAPI and viewed under a fluorescent microscope and photographed.

3.5 Flow Cytometry

1. Undifferentiated and differentiated cells were trypsinized and collected as single cell suspension. Two washes in PBS were given; cells were centrifuged at 1,000 × *g* for 5 mineach to collect the cell pellet.
2. Cell pellet was fixed in 4 % PFA for 20 min at 4 °C and processed immediately. 1 mL of PFA was used to fix 3×10^6 cells. Alternatively, the PFA fixed cells were stored at 4 °C and analyzed within 15 days of harvesting.
3. For analysis, the PFA fixed cells were centrifuged, PFA decanted out and the cell pellet washed twice with 1× PBS. The cell pellet was then resuspended in 2 mL FACS buffer. For staining, 0.1×10^6 cells were aliquoted in 1.5 mL microfuge tubes. Cells were then centrifuged, supernatant aspirated out and the cell pellet containing 0.1 million cells was resuspended in 100 μL FACS buffer. 0.1 Million cells each of unstained control, isotype control and the respective primary antibody were processed in identical manner in three separate microfuge tubes.
4. Primary antibodies were added to each cell suspension of 0.1 million cells and mixed well by pipetting. Primary antibody incubation was carried out at room temperature for 1 h. Concentration of the isotype control was kept same as that of the respective primary antibody.
5. Each of the samples was washed twice after the primary antibody incubation using FACS buffer. Again 100 μL of cell suspension was left at the bottom of the tube.

6. Respective secondary antibodies were added to the cell suspension of 100 μL from **step 5**, and incubation was carried out for 30–45 min at 4 °C in dark. Post secondary antibody incubation, each sample was washed with FACS buffer, centrifuged; supernatant aspirated leaving the stained cell suspension in 100 μL volume.
7. Each stained sample was stained in approximately 300 μL of FACS buffer, transferred to FACS tube kept in ice until analysis.
8. Samples were analyzed on a BD FACS Canto or any flow cytometer capable of excitation at 488 and 630 nm wavelengths. After excluding the dead cells and debris by applying forward scatter vs. side scatter gates, appropriate positive populations were obtained. Isotype controls were used for compensation controls and appropriate gating. Gated populations enabled quantitative comparisons of marker expression in samples collected from various steps of differentiation.
9. The data was analyzed using the FACS Diva software (BD Biosciences). 10,000 events were acquired per sample and results were represented in the form of histogram and/or dot plots.

3.6 In Vitro Functional Assay (Insulin and C-Peptide Assay)

1. Islet like clusters (ILC) in 3D form was cultured in low attachment 6-well dishes (Nunc) at a density of 15, 3D ILC per cm^2 for 48 h.
2. On the third day, the culture medium was changed to media with no glucose and then incubated overnight.
3. The next day, the cells were washed with Krebs–Ringer bicarbonate buffer for 2 h and treated with different concentrations of Glucose (3.3 and 16.7 mM) alone and also with combination with 10 mM Tolbutamide, 50 mM Nifedipine and 250 mM Diazoxide and incubated for 2 h respectively.
4. Culture supernatants were collected from different treatments. Insulin levels were estimated using human insulin ELISA kit and C-peptide levels were estimated using the C-peptide ELISA kit, both purchased from Mercodia, Sweden as per manufacturer's instructions.
5. Human plasma was run as a control along with the samples. Protein was estimated using the Bradford assay (Bio-Rad). Insulin concentration was normalized to the total amount of protein and expressed as mU/mg of total protein. C-peptide concentrations were expressed as pmol/mg of total protein.

3.7 In Vivo Preclinical Validation of In Vitro Derived Functional β Islets

1. NOD-SCID mice purchased from Jackson labs were bred, and male mice of 10 week old were used for the study. Total 12 mice were used, 6 control group and 6 transplanted group.
2. Streptozotocin stock was prepared in Citrate buffer, pH 4.5 immediately before administration.

3. All the animals were weighed and checked for blood glucose levels prior to STZ injections.
4. Animals were rendered diabetic with two doses of STZ, first dose 100 mg/kg body weight and second dose 40 mg/kg of body weight interspersed over a period of 72 h.
5. Diabetic state of the animals was confirmed by a steady state fasting glucose level of $\geq$300 mg/dL over a period of 1 week.
6. Diabetic animals were injected insulin till the transplantation of in vitro derived islets to prevent mortality.
7. On the day of transplantation, ~1,200 3D ILC were collected, centrifuged at a speed of 500 g and resuspended at 200 ILC/ 20 μL of **step 5** differentiation media. Surgeries were carried out under a laminar flow hood under aseptic conditions.
8. The animals, one at a time, were anesthetized using avertin injected intraperitonially at a dose of 10 μL/mg of body weight.
9. The animal was laid gently on a sterile dissection board with the ventral side facing up. The fur of the trunk portion was carefully shaved using an electric shaver.
10. One horizontal incision was made on the left side of the animal through which the kidney was gently pushed out on the surface of the skin. The kidney was kept hydrated by irrigation with PBS, applied using a wet earbud, during the surgery.
11. 20 μL of ILC suspension containing 200 3D ILC from **step 7** was gently placed underneath the kidney capsule using a 1 mL syringe with the bent needle. Leakage of the cells was prevented (please refer to Section 4.1 to troubleshoot the issues related to leakage of the cells). The control animals received 20 μL of **step 5** differentiation media without any cells.
12. The kidney was then gently pushed inside the abdominal cavity, the incision sealed with the help of glue. Antibiotic ointment was applied on the sealed incision.
13. The animals were then allowed to recover from anesthesia by placing the cage under an infrared lamp.
14. The animals were followed for blood glucose levels, body weight and mortality till 96 days post-transplantation.

3.8 Immunohistochemistry of Tissues

1. The animals were sacrificed and the kidneys were harvested and fixed in formalin-based fixative for 48 h. Only the area of interest was excised and the tissue sections were embedded into paraffin blocks (please refer to Section 4.1 to address the troubleshooting issues related to proper assessment of area of interest during sectioning).

2. Sections of 10 μm thickness were taken from taken from paraffin embedded tissue with the help of a microtome and prepared on to glass slides.
3. Tissues were then deparaffinized by incubation in xylene with 2–3 changes, 5 min each. Rehydration of the sections was done by passing them through different grades of alcohol (100, 95, 80, and 50 %, respectively).
4. The sections were rinsed in PBS. HIER (heat-induced epitope retrieval) through microwave irradiation was done for antigen retrieval in citrate buffer for 10 min.
5. Sections were blocked in 1 % BSA/PBS at room temperature for 1 h. Overnight incubation of 1:20 dilution of anti-insulin primary antibody was done at 4 °C.
6. After two brief washes, incubation with the FITC conjugated secondary antibody (as detailed in Tables S3 and S4) was done at a dilution of 1:500 for 1 h in dark.
7. Similarly staining was done for C-peptide.
8. After two washes for 5 min each at room temperature, the sections were mounted using mounting media and coverslips. The slides were then visualized and photographed in dark under a Zeiss fluorescence microscope.

4 Notes

Experiments	Problems	Probable reasons	Solutions
In vitro differentiation	Oct3/4 continues to persist after **step 2** of differentiation.	Incomplete initiation of differentiation.	Plate the EBs sparsely onto the Matrigel coated plates to ensure proper contact of the cells with the DE inducers.
In vitro differentiation	Cells show no inter-islet connections in **step 4** of differen tiation.	Incomplete β islet differentiation. Most of the cells might be at DE or PE stage.	Increase the duration of treatment by 3–4 days by 2D-ILC inducers. If the problem continues to persist, start a new batch of differentiation.

(continued)

(continued)

Experiments	Problems	Probable reasons	Solutions
In vivo islet transplantation	Absence of transplanted cells in the tissue sections.	Excision of incorrect area. Or, the cells were not transplanted properly and have leaked out during transplantation.	Ensure excising the correct area containing transplanted cells for immunocytochemical assessment. Ensure correct transplantation of cells underneath the kidney capsule using extra precautions and a good source of light during surgery.

1. Always ensure the health of pluripotent stem cells before using them for in vitro differentiation experiments. Healthy PSC can be identified by checking their morphology and sharp borders of PSC colonies with high Oct3/4 and telomerase activity.
2. Ensure a mycoplasma and endotoxin free MEFs and PSC cultures before starting the in vitro differentiation experiments.
3. Handle the cells gently.
4. Also, ensure a quick propensity of endodermal differentiation ($\geq$60 % Sox 17 positive cells) of the PSC cell line used for the study. Sox 17 positive cells can be obtained by 72 h treatment of the PSC lines directly plated onto Matrigel coated plates and treated with Activin A to a final concentration of 100 ng/mL.
5. Always maintain the hESC/PSC in low oxygen (3 %) incubator for maintaining healthy PSC with least oxidative damage to the cells.
6. Maintain primary cultures of MEFs in low oxygen (3 %) incubator.
7. Carry out the EBs differentiation as well as the entire differentiation under normoxic conditions in a regular CO_2 cell culture incubator.
8. Ensure $\geq$60 % of PDX-1 positive cells in the end of **step 3** of differentiation, or else, start a new batch of differentiation.

1. Do not proceed with the in vitro differentiation if the PSC lines are unhealthy.
2. Do not proceed further with the in vitro pancreatic differentiation if the cell lines used have low propensity for endodermal differentiation ($\leq$60 %).
3. Do not carry out the in vitro differentiation under hypoxic conditions.

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Generation of Corneal Keratocytes from Human Embryonic Stem Cells

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Abstract

Human Embryonic Stem Cells (hESC) offer an important resource as a limitless supply of any differentiated cell type of the human body. Keratocytes, cells from the corneal stroma, may have the potential for restoration of vision in cell therapy and biomedical engineering applications, but these specialized cells are not readily expanded in vitro. Here we describe a two-part method to produce keratocytes from the H1 hESC cell line. The hESC cells, maintained and expanded in feeder-free culture medium are first differentiated to neural crest cells using the stromal-derived inducing activity (SDIA) of the PA6 mouse embryonic fibroblast cell line. The resulting neural crest cells are selected by their expression of cell-surface CD271 and subsequently cultured as 3D pellets in a defined differentiation medium to induce a keratocyte phenotype.

Keywords: Cornea, Human embryonic stem cells, hESC, SDIA, Neural crest, Keratocyte, Stem cells

1 Introduction

1.1 The Need for Corneal Progenitor Cells

Vision loss and impairment due to corneal scarring affects millions of people worldwide. Currently, treatment is limited to corneal transplant, a procedure that requires donor tissue availability and which can be complicated by immunological rejection and graft failure. As a result, there is increased interest in therapies that decrease dependency on donor tissue such as cell-based therapies, and in bioengineering of corneal tissue. Corneal scarring occurs in the stroma, the transparent connective tissue which constitutes >90 % of corneal thickness. Cells of the stroma, the keratocytes, are a quiescent population of neural crest-derived mesenchymal cells responsible for secretion of the highly specialized transparent tissue of the stroma. Keratocytes are limited in abundance and change phenotype when expanded in culture to fibroblasts and myofibroblasts, cells that secrete non-transparent corneal scar tissue (1–5). Thus, access to these cells for regenerative approaches is only possible using cells with the potential to differentiate into keratocytes, i.e., stem or progenitor cells.

1.2 Generating Keratocytes from Stem Cells

Directed differentiation of hESC to any cell fate within the body may lead to remarkable advances in tissue regeneration via cell therapy. To realize this goal, conditions for directed differentiation must be established to generate pure, high quality, functional populations of the cell type of interest. We have developed a method for generating corneal keratocytes, the functional cell type of the corneal stroma (6).

During embryonic development keratocytes are derived from neural crest stem cells (NCSC) that migrate from the neural tube. To recapitulate this in vitro, hESC are differentiated to NCSC via stromal-derived inducing activity (SDIA) by coculture with the murine PA6 cell line (7, 8). NCSC are then sorted to select for cells expressing the NCSC cell surface marker CD271 (NGFR, nerve growth factor receptor; $p75^{NTR}$). $CD271^{+}$ cells are subsequently cultured and expanded in a serum-free medium that preserves the neural crest phenotype. When the desired cell number is reached, $CD271^{+}$ cells are cultured for 2 weeks as pellets in keratocyte differentiation medium (KDM). Upregulation of genes present in keratocytes such as CHST6, PTGDS, and the keratocyte-specific proteoglycan keratocan can be used to confirm differentiation of hESC to corneal keratocytes.

2 Materials

hESC line WA01 (H1) (WiCell, Madison, WI) (We expect the same approach to be applicable to other lines such as H9.) (*see* **Note 1**).

Matrigel (BD Biosciences).

Thaw at 4 °C and aliquoted into individual samples containing 270–350 μL (based on the Product Certificate of Analysis). The samples are stored at 80 °C and thawed at 4 °C before dilution to working strength.

mTeSR-1 (StemCell Technologies).

StemPro EZPassage Tool (Life Technologies).

Tissue culture dish 100 × 15 mm TC treated.

PA6 Cell Line (Riken Bioresource Center Cell Bank, Japan).

Gelatin.

TrypLE Express.

PA6 Culture Medium; 90 % MEM-alpha, 10 % fetal bovine serum.

Induction Medium; 90 % BHK21-medium/Glasgow modified Eagle's medium, 2 mM glutamine, 10 % knockout serum replacement, 1 mM sodium pyruvate, 0.1 mM nonessential amino acid solution, 0.1 mM β-mercaptoethanol, 100 IU/mL penicillin, 100 μg/mL streptomycin.

Accutase.

Cell strainer, 70 μm.

Wash Buffer: phosphate-buffered saline (PBS), 0.5 % fetal bovine serum (FBS), 0.1 mM ethylenediaminetetraacetic acid (EDTA).

CD271 Microbead Kit (Miltenyi Biotec) containing MACS MS Column and MACS Cell Separator.

PO/L/F (poly-L-ornthine/laminin/fibronectin) Coated Plates; Poly-L-ornithine 15 μg/mL, Laminin 1 μg/mL, Fibronectin 10 μg/mL, to coat P/O/L/F plates, first coat with poly-L-ornithine overnight at 4 °C. Rinse with PBS twice the following day. Combine and add laminin (1 μg/mL) and fibronectin (10 μg/mL) and incubate for 2 h at 37 °C.

DME/F12.

N2 Supplement (Life Technologies).

10 ng/mL FGF-2.

10 ng/mL EGF.

Advanced DMEM.

0.1 mM ascorbic acid-2-phosphate (Sigma).

10 ng/mL FGF-2.

2.1 Stock Solutions

Reagent	Concentration	Solvent	Storage
FGF-2	10 μg/mL	Sterile water	−80 °C
Ascorbic acid-2-phosphate	500 mM	Sterile water	−80 °C
Poly-L-ornithine	1 mg/mL	Sterile water	−80 °C
Laminin	1 mg/mL	Sterile water	−80 °C
Fibronectin	1 mg/mL	Sterile water	−80 °C
EDTA	500 mM	Sterile water	Ambient
Penicillin/streptomycin		Sterile water	−20 °C
Gentamycin		Sterile water	4 °C
Gelatin	1 %	Sterile water	4 °C
β-mercaptoethanol		Sterile water	−20 °C

3 Methods

3.1 Expansion of hESC Cultures

1. hESC are cultured on Matrigel-coated plates in mTeSR-1 basal medium (9, 10).
2. To coat a 100 mm culture dish, frozen (*see* above) aliquots of Matrigel are thawed at 4 °C and diluted in 25 mL DMEM/F12 with mixing by inversion multiple times.

3. Add ~8 mL diluted Matrigel + DMEM/F12 mixture to a 100 mm dish. Allow the proteins to attach to the plastic at room temperature for at least 1 h (*see* **Note 2**).
4. Undifferentiated hESC either cryopreserved or from passage (below) grow on Matrigel-coated dishes in flattened colorless round colonies, 1–2 mm in diameter, with well-defined edges, phase-bright colony centers, and minimal overt spontaneous differentiation.
5. mTeSR medium is changed daily.
6. The cultures are expanded every 5–7 days or when the colonies become so large that they begin to touch one another. Without removing medium, a StemPro EZ Passage Tool in is rolled over the plate twice in an orthogonal pattern to fragment hESC colonies. It is important that cells remain in discreet multicellular aggregates of 80–150 μm. Smaller aggregates (<60 μm) may exhibit decrease viability and induce spontaneous differentiation (*see* **Note 3**).
7. The colony fragments are centrifuged 200 × *g* for 2 min and the pelleted fragments are resuspended in enough medium for a 4× expansion.
8. hESC are cultured in a humidified incubator at 37 °C in 5 % CO_2. Replace the medium daily.
9. To make a 0.1 % gelatin solution, first make a stock solution of 1 % gelatin by dissolving 1 g gelatin in 100 mL 1× PBS. Autoclave to dissolve and sterilize the solution.
10. Dilute the 1 % gelatin stock solution 1:10 in sterile 1× PBS to make a 0.1 % solution.

3.2 Preparation of PA6 Cells for SDIA

1. Add 10 mL of a 0.1 % gelatin solution to each 100 mm dish. Allow the protein to attach to the plates for 2 h at room temperature.
2. Rinse the plates twice with 1× sterile PBS.
3. Plate PA6 cells at approximately 5×10^4 cells/cm^2 on 0.1 % gelatin coated plates.
4. PA6 cells are grown in a humidified incubator at 37 °C in 5 % CO_2. Passage the cultures 1:5 by trypsinization when they become 80 % confluent.

3.3 Induction of NCSC Differentiation by PA6/hESC Coculture

1. Overgrown and differentiated hESC colonies are first manually removed from culture plates using a glass pipette. Overgrown colonies are identified as pigmented (usually a brown coloration visible without a microscope) and raised. They are removed by scraping with the tip of a Pasteur pipette or similar sterile sharp implement. *see* **Note 2**.

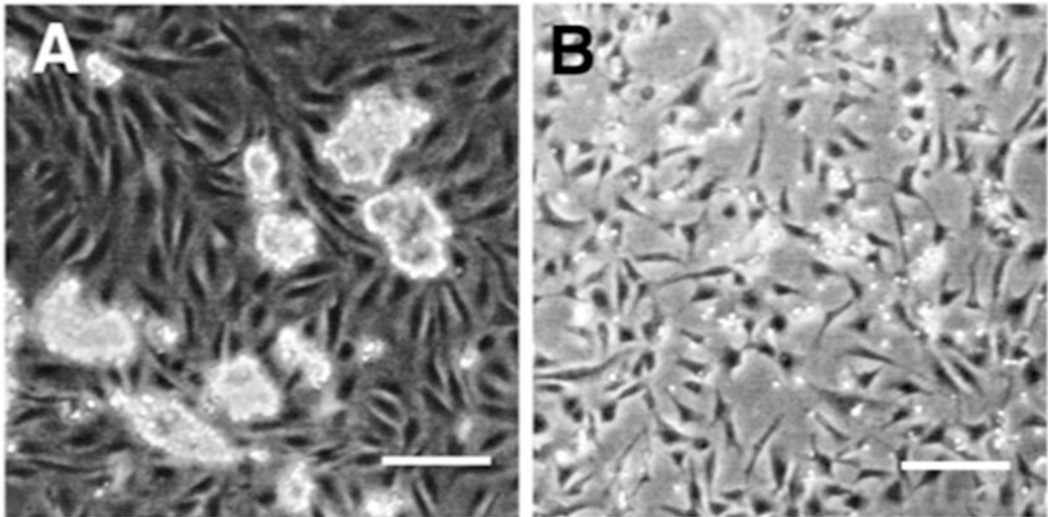

Fig. 1 hESC coculture and CD271$^+$ cells after isolation. (**a**) hESC coculture on PA6 cell monolayer. hESC retain colony-like morphology while the underlying PA6 cells remain confluent and spindle-like. (**b**) CD271$^+$ Cells grown in serum free medium after isolation via MACS cell separator. Scale bars are 200 μm. Figure from ref. (6)

2. Undifferentiated hESC colonies are then sectioned and collected using the StemPro EZPassage tool and collected in a 50 mL conical tube.
3. Gently wash colonies in 1× PBS and spin for 5 min at 200 × *g*. Be careful to keep colonies as clumps and do not allow cells to become a single-cell suspension.
4. Resuspend the loose pellet in *Induction Medium*.
5. hESC colonies are added dropwise to 95 % confluent PA6 cells at approximately 9,000 colonies per 100 mm dish. The coculture plate is then incubated at 37 °C in 5 % CO_2 for 6 days without changing medium (Fig. 1).

3.3.1 NCSC Isolation

1. Add 1 mL Accutase per 100 mm plate and rock back and forth to cover entire plate.
2. Incubate at ambient temperature observing the cultures using an inverted microscope every 5 min to monitor detachment of the hESC colonies from the PA6 monolayer.
3. Collect the released colonies by washing the plate with 5 mL wash buffer (0.5 % FBS, 0.1 mM EDTA in PBS). Triturate the colonies with a 10 mL pipette to achieve a single cell suspension and remove aggregates with a 70 μm cell strainer.
4. Count the cells. Each MS column will accommodate up to 2×10^8 cells.
5. Pellet up to 2×10^8 cells by centrifuging at 500 × *g* for 10 min.

6. Resuspend the cells in 80 μL wash buffer and add 10 μL FcR Blocking Reagent (Miltenyi Kit) and 10 μL APC-labeled anti-CD271 antibody (Miltenyi Kit). Incubate for 15 min in the shelf of a 4 °C refrigerator.
7. Add 1 mL of wash buffer and pellet cells as in **step 5**.
8. Resuspend the pellet in 70 μL wash buffer. Add 10 μL FcR blocking reagent followed by 20 μL Anti-APC MicroBeads. Incubate for 15 min in the refrigerator.
9. Wash the cells as in **step 5**.
10. Resuspend the cells in 500 μL wash buffer.
11. To prepare MS column, place the column in the Miltenyi Cell Separator magnet and rinse the column with 2 × 500 μL portions of wash buffer (*see* **Note 4**).
12. Replace waste tube with a 12 × 75 collection tube labeled "Flow Thru".
13. Slowly pipette 500 μL filtered cell suspension to the column, be careful not to introduce bubbles which clog the column.
14. Wash column 2× with 500 μL wash buffer.
15. Remove MS column from magnet and place over a new collection tube labeled "Bound". Elute bound cells with 500 μL wash to column.
16. Rinse the column with two more 500 μL portions wash buffer.
17. Cells in both tubes are centrifuged, resuspended in 1 ml Wash Buffer, and counted. The samples are taken for RNA isolation.
18. Expression of CD271 in the "Bound" population is confirmed by flow cytometry (Fig. 2).
19. To improve the proportion of CD271+ cells, the isolation can be repeated immediately by repeating the magnetic column-based isolation.
20. Neural crest gene mRNA expression is confirmed by qPCR (Fig. 3; Table 1).
21. The "Bound" cell population is expanded on PO/L/F coated culture dishes at 10^4 cells per cm^2 in N2 medium.

3.4 Keratocyte Differentiation

1. After 1 week culture in N2 Medium NCSC are harvested with TrypLE express.
2. $2 \times 10^{5+}$ cells at are collected by centrifugation at 423 × *g* for 5 min to form a pellet.
3. These are cultured for 2 days in DMEM/F12 with 2 % FBS at 37 °C in 5 % CO_2.
4. After 2 days, the medium is changed to KDM, being careful not to disturb the pellet.

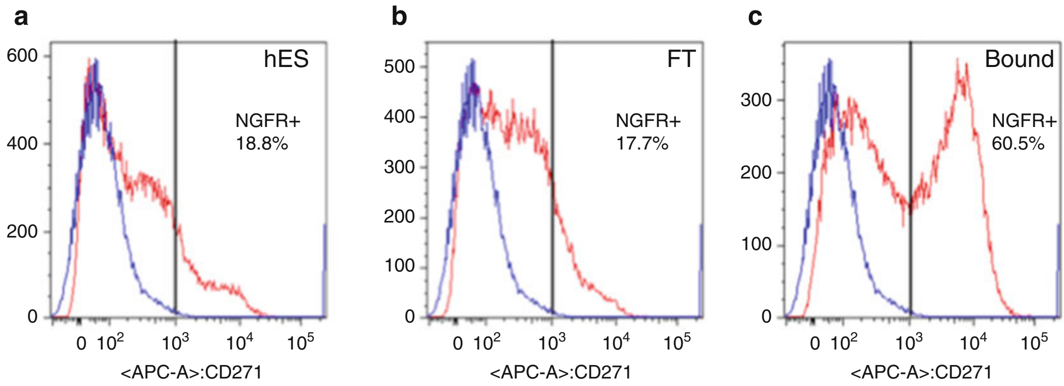

Fig. 2 Flow cytometry verification of CD271 MACS. MACS separation of CD271 from PA6 cells after 6 days of coculture. (**a**) CD271 is expressed by 18.8 % of hESC cells before coculture. (**b**) The flow through (FT) fraction contains 17.7 % CD271+ cells. (**c**) The bound fraction contains 60.5 % CD271+ cells. The *vertical black bar* marks the population with <0.1 % nonspecific staining. The *blue line* shows staining with isotype-matched control antibody. The *red line* shows CD271-APC stained cells. Percentage of stained cells is shown. Figure from ref. (6)

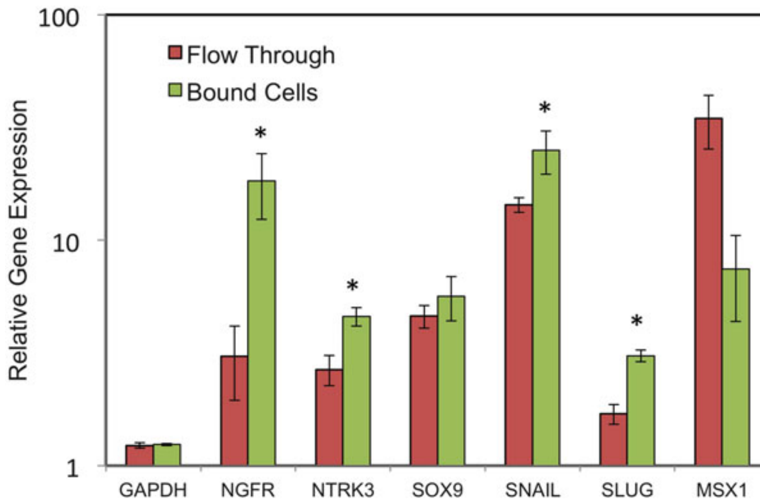

Fig. 3 Neural Crest Gene Expression of $CD271^{+}$ Sorted Cells. Neural Crest Genes are Upregulated after PA6 Coculture. Expression of the neural crest genes NGFR, NTRK3, SOX9, SNAI1, SLUG, and MSX1 was measured after 6-days of hES:PA6 coculture. Bound ($CD271^{+}$) cells were compared to the flow through ($CD271^{+}$). Error bars represent standard deviation of triplicate reactions. *Asterisks* show significant ($p < 0.05$) increase in expression of NGFR, NTRK3, SNAI1, and SLUG by bound cells compared to the flow-through population as determined by Student's *t*-test. Figure from ref. (6)

Table 1
PCR primer sequences used

Gene	Forward primer	Reverse primer
GAPDH	TGTTGCCATCAATGACCCCTT	CTCCACGACGTACTCAGCG
NGFR	CCTACGGCTACTACCAGGATG	CACACGGTGTTCTGCTTGTC
NTRK3	TCCGTCAGGGACACAACTG	GCACACTCCATAGAACTTGACA
SOX9	GCCAGGTGCTCAAAGGCTA	TCTCGTTCAGAAGTCTCCAGAG
SNAI1	AATCGGAAGCCTAACTACAGCG	GTCCCAGATGAG CATTG GCA
SLUG	AAGCATTTCAACGCCTCCAAA	AGGATCTCTGGTTGTGGTATGAC
MSX1	CTCCGCAAACACAAGACGAAC	CACATGGGCCGTGTAGAGTC
AQP1	CTG CACAGGCTTGCTGTATG	TGTTCCTTGGGCTGCAACTA
ALDH	CATTGGCACCTGGAACTACC	GGCTTGAGGACCACTGAGTT
B3GNT7	CCTCMGTGGCTGGACATCT	ACGAACAGGTTTTCCTGTGG
KERA	ATCTGCAGCACCTTCACCTT	CATTGGAATTGGTGGTTTGA
PTGDS	CGGGGTCCCTCGGCTCCTAC	CTGGGGGTCTGGGTTCGGCT

Table from ref. (6)

5. 50 % of the medium is replaced every 3 days for 2 weeks (11).
6. After 2 weeks, keratocyte phenotype can be observed by qPCR (Fig. 4; Table 1) for markers such as Aquaporin 1, Aldehyde Dehydrogenase 3A1, B3GNT7, Keratocan, and Prostaglandin D2 Synthase, or by immunoblotting to detect the stroma-specific keratan sulfate proteoglycans with antibodies to keratocan and keratan sulfate glycosaminoglycan (Fig. 5).

4 Notes

1. Use of hESC cell lines is available via license and is restricted so that only some lines are available for in vivo experimental use. Investigators should consult the NIH Stem Cell Registry http://grants.nih.gov/stem_cells/registry/current.htm to inform themselves about these restrictions.
2. Matrigel-coated plates can be stored at 4–8 °C for up to 2 weeks if not used immediately after coating by leaving Matrigel solution on the plate and sealing with Parafilm to keep from drying. Before using, let plate sit at room temperature for 1 h.
3. Manipulations of hESC need to be carried out in BSL2 containment. This will require using an inverted microscope with a low-power objective (typically 2×) in a laminar flow tissue culture hood. Visualization of the colonies is most conveniently

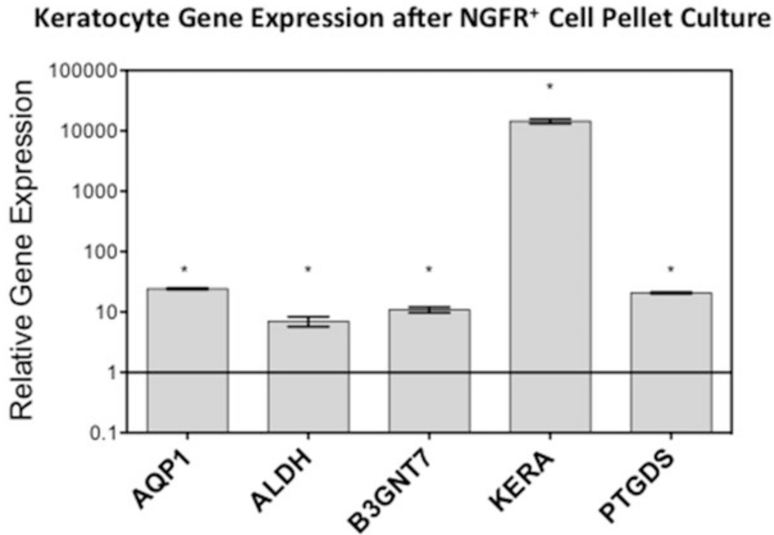

Fig. 4 Keratocyte gene expression after differentiation. Keratocyte Gene Expression after CD271$^+$ Pellet Culture. After CD271$^+$ cells were expanded as monolayers in N2 medium there were grown as pellets in keratocyte differentiation medium for 2 weeks. Keratocyte gene expression was significantly upregulated 2 weeks after pellet culture compared to CD271$^+$ cells. *Asterisks* represent significant ($p < 0.05$) upregulation as determined by Student's *t*-test. Error bars represent standard deviation. Figure from ref. (6)

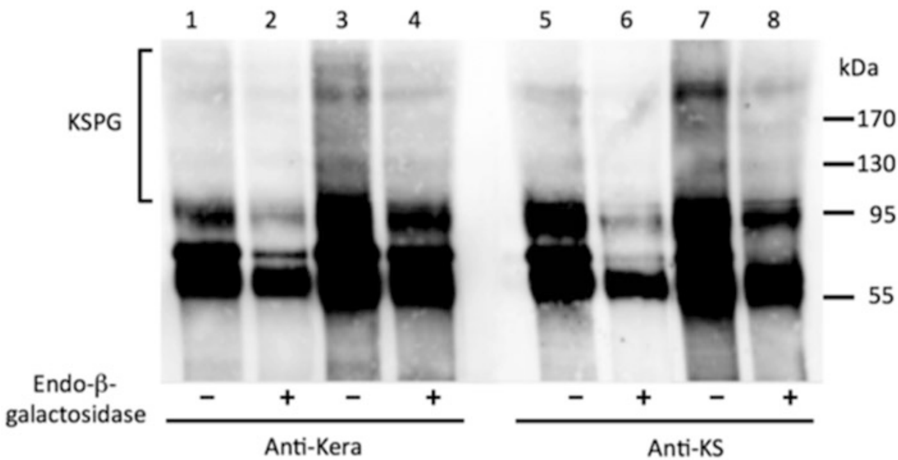

Fig. 5 Immunoblot of keratocan and keratan sulfate. Keratan sulfate proteoglycan secretion after pellet culture. Differentiated cells secret corneal keratan sulfate proteoglycans after 3 weeks of pellet culture. Medium from cultures was taken before (Lanes 1, 2, 5, 6) or after (Lanes 3, 4, 7, 8) 3 weeks of CD271$^+$ pellet culture in KDM. Proteoglycan fractions were biotin-labeled and immune-precipitated with antibodies against Keratocan (Anti-Kera, Lanes 1–4) or Keratan sulfate glycosaminoglycan (KSPG) (Anti-KS, Lanes 5–8). Both Keratocan and KSPG are present after 3 weeks of pellet culture in KDM. Figure from ref. (6)

effected using a USB eye-piece CCD camera (such Dino-Eye Series AM4023, BigC.com, Torrance, CA) to provide real-time video to a laptop computer outside the sterile field.

4. Whenever MS columns are washed, it is imperative that no bubbles are introduced as this will cause the column to clog and cell sorting will be ineffective.

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Maintenance, Transgene Delivery, and Pluripotency Measurement of Mouse Embryonic Stem Cells

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Abstract

This chapter describes standard techniques to (1) maintain mouse embryonic stem cell culture, (2) deliver transgenes into mouse embryonic stem cells mediated by electroporation, nucleofection, lipofection, and retro/lentiviruses, and (3) assess the pluripotency of mouse embryonic stem cells. The last part of this chapter presents induction of random cell differentiation followed by the alkaline phosphatase and embryoid body formation assays, immunofluorescence microscopy, and the teratoma formation assay.

Keywords: Mouse embryonic stem cells (mESCs), Electroporation, Nucleofection, Lipofection, Retrovirus, Lentivirus, Self-renewal, Pluripotency, Alkaline phosphatase, Immunofluorescence microscopy, Embryoid body, Teratoma

1 Introduction

Mouse embryonic stem cells (mESCs) are dependent on leukemia inhibitory factor to sustain their self-renewal (1, 2). Because of this simple rule, mESCs have been offering a powerful platform to study gene functions associated with self-renewal and differentiation through the analysis of gain-of-function or loss-of-function phenotypes. This chapter describes maintenance of standard mESC culture and gene delivery-based approaches to evaluate gene functions in mESCs. In particular, the following four methods are presented: electroporation-, nucleofection-, lipofection-, and retro/lentivirus-based gene delivery (Table 1). Electroporation and nucleofection offer simple and easy delivery of plasmids into mESCs, although require special instruments. Lipofection provides flexibility with respect to the size of culture, whereas has a limited capacity to deliver large plasmids and a large number of plasmid molecules inside mESCs. The advantage of virus-based vectors is to deliver a gene expression cassette as it is (without loss of DNA ends). However, subcloning of a gene expression cassette into a viral vector requires screening of a large number (200<) of transformed *E. coli* to identify a correct vector with the cassette ligated. In addition, due to the use of a viral packaging cell line derived

Table 1
Comparison of common DNA delivery methods

Method	DNA (μg)	# of mESCs needed ($\times 10^6$)	Instrumentation	Integration site	Other comments
Electroporation	10 (or more)	1–20	Electroporation system (e.g., Gene Pulser, BTX ECM 200)	Random, multiple	
Nucleofection	1–10	2–5	Nucleofector (Lonza)	Random, multiple	No flexibility to adjust conditions
Lipofection	2	1 or less	No special instrument	Random, multiple	The size of culture is flexible. The efficiency is limited by the vector size
Retro/lentivirus	30–40	3	Rocker platform Ultracentrifuge	Active transcription sites (45), can be single integration depending on the multiplicity of infection (MOI), but generates insertional mutagenesis	The transgene expression cassette will be delivered as it is. Building a proviral vector requires screening of hundreds of transformed bacterial colonies

from human cells, the work needs the biosafety level (BSL) 2 containment and prior approval from the institutional biosafety committee (IBC). Importantly, retro/lentiviruses will land at genomic sites with active transcription, which will highly likely result in insertional mutagenesis (Table 1). Although other three methods similarly disrupt the original context of the host genome and transgene expression is under the influence of the transcriptional activity of neighborhoods, the insertion of plasmids will be more random and can be in intergenic regions.

2 Materials

2.1 Maintenance of Mouse Embryonic Stem Cells

1. Embryonic day 12.5–13.5 embryos resulted from natural mating of C57BL/6 (e.g., the Jackson Laboratory) or CD1/ICR (e.g., Charles River Laboratories) mice to produce feeders (*see* **Note 1**).
2. 10× Phosphate-buffered saline (PBS): 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 20 mM KH_2PO_4. Ultrapure H_2O (e.g., Milli-Q H_2O) should be used to dissolve these salts. Autoclaved and stored at room temperature (RT). One part of 10× PBS should be diluted with nine parts of ultrapure H_2O to prepare 1× PBS for routine use. Store 1× PBS at RT.
3. 0.1 % gelatin in 1× PBS: Add gelatin (type A from porcine skin, Sigma G1890) to 1× PBS at 0.1 % and autoclave it. Store at 4 °C.
4. Gelatin-coated tissue culture dishes and multi-well plates: Add 0.1 % gelatin in 1× PBS to cover the surface of tissue culture dishes and plates as shown in Table 2. These dishes and plates can be prepared in advance and stored at 4 °C with the gelatin solution left inside. Aspirate 0.1 % gelatin immediately after the entire surface is covered (no need to incubate it), or before plating mESCs.
5. TrypLE™ Express: This ready-to-use recombinant trypsin solution (Life Technologies, e.g., 12604-021) is quite stable for several months when stored at 4 °C. No need to aliquot it.

Table 2
List of solutions and volumes needed per culture size

Size of culture	0.1 % gelatin in PBS (ml)	Medium (ml)	PBS (ml)	TrypLE™ Express (ml)
3.5-cm dish	1	2	1	0.5
6-cm dish	1.5	4	1	1
10-cm dish	2.5	15	1–2	1.5
24-well plate	0.2	1	0.5	0.2
6-well plate	1	2	1	0.5

6. Fetal bovine serum (FBS) qualified for ESC culture (e.g., Life Technologies 16141-079; Gemini Bio-products 100-500; *see* **Note 2**). If a bottle of FBS is used up within 3 months after thawed, it can be stored at 4 °C. Otherwise, FBS should be aliquoted and stored at −20 °C or below.
7. Dimethyl sulfoxide (DMSO), cell culture tested (e.g., Sigma D2650).
8. Mitomycin C (e.g., Sigma M4287): Immediately before use, it should be reconstituted using 5 ml 1× PBS (40× solution) and filter-sterilized. One vial of 2 mg is good to treat eight to nine 15-cm dishes at 10 μg/ml, 20 ml per dish. Discard the leftover (*see* **Note 3**).
9. mESCs: A variety of mESCs are available through ATCC. R1, J1, RW.4, and E14TG2a mESCs are proven to be germ-line competent.
10. Dulbecco's modified eagle medium (DMEM), High-glucose (e.g., Life Technologies 11960-069): Store at 4 °C.
11. 200 mM GlutaMAX™ supplement, 100× (e.g., Life Technologies 35050-061): Store at 4 °C.
12. 100 mM Sodium pyruvate, 100× (e.g., Life Technologies 11360-070): Store at 4 °C.
13. 10 mM MEM nonessential amino acids (NEAA), 100× (e.g., Life Technologies 11140-050): Store at 4 °C.
14. Penicillin–streptomycin, 100× (e.g., Sigma P4333): Penicillin–streptomycin needs to be aliquoted (12 ml each, for two bottles) and stored at −20 °C. However, penicillin and streptomycin are not essential for mESC culture if good sterile technique is carried out.
15. 2-mercaptoethanol (2-ME; e.g., Sigma M3148): Store at RT. Its working concentration is 0.1 mM.
16. Leukemia inhibitory factor (LIF; EMD Millipore ESG1107, 1×10^7 units/ml): LIF is quite stable for a couple of years when stored at 4 °C. No need to aliquot it. Its working concentration is 1,000 units/ml.
17. ESC culture media: To prepare complete ESC culture medium, a bottle of high-glucose DMEM (500 ml) will be supplemented with 93 ml FBS, 6 ml 200 mM GlutaMAX™ supplement, 6 ml 100 mM sodium pyruvate, 6 ml 10 mM MEM NEAA, 6 ml 100× penicillin–streptomycin, 4.2 μl 2-ME, and 60 μl LIF. Incomplete ESC culture medium that lacks LIF is referred to as LIF− medium hereafter.
18. PCR primers: MCGpF11 (ACA CCA TGG GAG YTG GTA AT), MCGpR21 (GCA TCC ACC AWA WAC YCT T),

R16-2 (GTG SGG MTG GAT CAC CTC CT), MCGpF2 (GTT CTT TGA AAA CTG AAT).

19. 50× Tris–Acetate–EDTA (TAE) buffer: 48.5 g Tris, 11.4 ml glacial acetic acid, and 3.72 g EDTA 2Na $2H_2O$ (or 20 ml 0.5 M EDTA, pH 8.0) are mixed and the volume is adjusted to 200 ml with deionized H_2O. Autoclave and store it at RT.
20. 1 % agarose in 0.5× TAE: Weigh molecular biology-grade agarose (e.g., Sigma A9539) into a glass bottle with a plastic screw cap and accordingly dilute 50× TAE 100-fold with deionized H_2O in the bottle to make 1 % agarose in 0.5× TAE. Close the cap and microwave the agarose–TAE mix. Once the liquid starts boiling, stop and reduce the power of microwave, or repeat brief microwave for a few seconds to prevent explosion of the content, until agarose is completely melted and becomes homogeneous liquid. When the agarose is cooled down and the bottle can be held by hands for several seconds, add a fluorescent dye for detection of DNA (e.g., GelGreen, Biotium, with Dark Reader®, Clair Chemical Research, Inc.).

2.2 Delivery of a Transgene into Mouse Embryonic Stem Cells

1. Either a plasmid or a retro/lentiviral vector that harbors a transgene of interest: Ligating a DNA fragment that encodes a transgene into a vector can be done by standard molecular cloning techniques. Typically subcloning of a transgene into a viral vector requires screening of a couple of hundreds of transformed *E. coli* (e.g., HB101 or Stbl2) by colony hybridization due to the recombination between two viral arms (i.e., long terminal repeats, LTR). For lentiviral vectors, the following three plasmid vectors that separately encode minimum but essential viral proteins are required for packaging; pLP1, pLP2, and pLP/VSVG.
2. Plasmid purification kit (e.g., Qiagen, 12662).
3. Restriction enzyme (e.g., New England Biolabs).
4. 3 M sodium acetate, pH 5.2: Ultrapure H_2O should be used to dissolve sodium acetate. The pH needs to be titrated with glacial acetic acid. Autoclave and store it at RT.
5. 0.8 % agarose in 0.5× TAE (*see* above).
6. 1× PBS.
7. 4-mm cuvettes for electroporation.
8. Transfer pipettes, 6 ml (e.g., Sarstedt 86.1175.020).
9. Nucleofection kit for mESCs (Lonza VAPH-1001).
10. Antibiotics: Powdered forms of G418 sulfate (e.g., Life Technologies 11811-023), puromycin dihydrochloride (e.g., Sigma P8833), and blasticidin S HCl (e.g., A.G. Scientific B-1247) can be obtained and reconstituted with PBS (stock solutions

can be 100 mg/ml for G418 and the others, 10 mg/ml). To increase the solubility of G418 sulfate, 10 N NaOH can be used. Reconstituted antibiotics need to be filter-sterilized, aliquoted, and stored at −20 °C (*see* **Note 4**).

11. Lipofection reagent (FuGENE® HD, Promega; *see* **Note 5**).
12. Virus packaging cell line (e.g., Phoenix™ cells (3, 4) and HEK293T cells (5, 6)).
13. 14 ml round-bottom tubes (e.g., Falcon 35-2059).
14. 2 M $CaCl_2$: Ultrapure H_2O should be used to dissolve $CaCl_2$. Filter-sterilize and store it at 4 °C.
15. 2× Hepes-buffered saline (2× HeBS): 280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM (*d*)-glucose, 50 mM HEPES. Ultrapure H_2O should be used to dissolve these salts. The pH should be adjusted to exactly pH 7.00 with 0.5 N NaOH. Filter-sterilize, aliquot, and store it at −20 °C.
16. 4 mg/ml polybrene (1,000× stock): Dissolve hexadimethrine bromide (Sigma H9268) in ultrapure H_2O to prepare 4 mg/ml stock. Filter-sterilize and store it at 4 °C. Its working concentration is 4 μg/ml.
17. V-bottom 96-well plates.
18. 2× freezing medium: Freshly mix one volume of DMSO (e.g., Sigma D2650), one volume of FBS and three volumes of complete ESC culture medium.
19. H_2O-washed mineral oil: Open a fresh bottle of mineral oil (e.g., Sigma M8410 or ones available at a local pharmacy) under a biosafety cabinet. Mix mineral oil and ultrapure H_2O 5:1 in a sterile manner. This results in reducing toxic substances in mineral oil (7). Store it at RT.

2.3 Measurement of the Pluripotency of Mouse Embryonic Stem Cells

1. 1 % Fast Violet B salt: Mix fast Violet B salt (Sigma F1631) with distilled H_2O at 1 %. Store at −20 °C. No need to aliquot.
2. 1 % Naphthol AS-MX phosphate: Mix naphthol AS-MX phosphate disodium salt (Sigma N5000) with 100 mM Tris–HCl, pH 9.0 at 1 %. Store at −20 °C. No need to aliquot.
3. Substrate for alkaline phosphatase reactions: To prepare 10 ml, first mix 0.1 ml 1 % Naphthol AS-MX phosphate, 0.2 ml 5 M NaCl, 1 ml 1 M Tris–HCl, pH 9.0, 0.5 ml 1 M $MgCl_2$, and 7.96 ml ultrapure H_2O. This mixture can be stored at −20 °C. Immediately before use, add 0.24 ml 1 % Fast Violet B salt and mix well.
4. 4 % paraformaldehyde (PFA) in 1× PBS: Dissolve PFA (e.g., Sigma 158127) in distilled H_2O to make 8 % stock solution. Heating it at 50–60 °C and adding a few drops of 1 N NaOH into it will increase the solubility of PFA. Do not add too much

NaOH or a neutral pH cannot be maintained when 4 % PFA is reconstituted into 1× PBS. Aliquot and store 8 % PFA at −20 °C. To prepare 4 % PFA in 1× PBS, one part of 10× PBS is mixed with four parts of distilled H_2O and five parts of 8 % PFA. Store reconstituted 4 % PFA at 4 °C and use it within a week. Because PFA is a carcinogen, it should be collected as a hazardous waste.

5. 2 % skimmed milk in 1× PBS with 0.1 % Tween 20/Triton X-100 (PBSMT): Nonfat skimmed milk available at a grocery store can be used. Either Tween 20 (e.g., Sigma P9416) or Triton X-100 (e.g., Sigma T8787) can be used to permeabilize cell membrane. Due to the viscosity, these reagents can be handled better with a syringe. Store at 4 °C for a couple of weeks or bacterial growth will occur.
6. Primary antibodies for immunofluorescence microscopy (IF): for validation of mESC pluripotency, anti-human Oct4 monoclonal antibody (Santa Cruz Biotechnology sc-5279), anti-SSEA1 monoclonal antibody (Developmental Studies Hybridoma Bank MC-480 or Santa Cruz Biotechnology sc-21702), anti-mouse Nanog polyclonal antibody (R&D systems AF2729), and anti-human Sox2 polyclonal antibody (Santa Cruz Biotechnology sc-17320); for validation of teratoma: anti-nestin monoclonal antibody (Developmental Studies Hybridoma Bank Rat-401 or Sigma SAB4200347), anti-α-fetoprotein polyclonal antibody (Santa Cruz Biotechnology sc-8108), and anti-α-smooth muscle actin polyclonal antibody (Abcam ab5694).
7. Fluorophore-conjugated secondary antibodies that recognize whole antibody molecules of animal species in which primary antibodies used are raised (e.g., Alexa Fluor® 488-conjugated goat anti-mouse IgG, Life Technologies A11001). Cross-reactivity of secondary antibodies must be tested first if more than two secondary antibodies are simultaneously used.
8. 500 μg/ml DAPI (1,000× stock): Use distilled H_2O to dissolve DAPI (e.g., Sigma D9542) or DAPI will not dissolve in 1× PBS at this concentration. Aliquot and store it at −20 °C for long-term storage, although this stock is stable at 4 °C for several months. Cover them with aluminum foil. The working concentration of DAPI is 0.5 μg/ml in 1× PBS.
9. 0.3 mg/ml Type IA collagen in 1× PBS: Dilute 3 mg/ml Cellmatrix Type I-A (Nitta Gelatin Inc. 631-00651, available from Wako Pure Chemical Industries, Ltd.) tenfold with 1× PBS. Store it at 4 °C (*see* **Note 6**).
10. Immunocompromised mice (e.g., nonobese diabetic mice with severe combined immunodeficiency disease (NOD/SCID mice) from the Jackson Laboratory).

11. Paraplast Plus® (e.g., Sigma P3683).
12. 10 mM sodium citrate buffer pH 6.0: To prepare 200 ml 50 mM stock solution, dissolve 399.2 mg citric acid monohydrate (e.g., Sigma C0706) and 2.382 g sodium citrate tribasic dehydrate (e.g., Sigma C8532). Store it at RT. One part of 50 mM sodium citrate should be diluted with four parts of distilled H_2O before use (8).
13. Histological staining solutions: 0.05 % Alcian Blue 8GX (e.g., A3157, Sigma) in 5 % acetic acid; Mayer's hematoxylin (e.g., 26043, Electron Microscopy Sciences); 1 % eosin Y (e.g., 26051, Electron Microscopy Sciences). Store them at RT.
14. Histo-Clear II (i.e., safe alternative to xylene).
15. Permount for mounting histologically stained tissue sections.

3 Methods

Unless otherwise specified, routine maintenance of cell culture should be carried out under sterile conditions inside the biosafety cabinet. The work surface should be sterilized with UV lamps for a few min, followed by spraying 70 % EtOH. Before starting work, culture media should be warmed at RT for a couple of hours or 37 °C for 30 min.

3.1 Maintenance of Mouse Embryonic Stem Cells

3.1.1 Isolation of Mouse Embryonic Fibroblasts (i.e., Feeders; See **Note 1**)

1. Set up a mating of an appropriate mouse strain (e.g., C57BL6 or CD1/ICR) and harvest embryos at embryonic day 12.5–13.5 (13th to 14th day after the presence of a vaginal plug is confirmed; *see* **Note 7**).
2. Euthanize a pregnant female mouse, wipe its abdomen with 70 % ethanol, and place the animal under the biosafety cabinet.
3. Pinch and pull the skin and make a small incision on it using a pair of autoclaved fine dissection scissors. Tear the skin by hands along the anterior–posterior axis.
4. Pull the peritoneum using autoclaved Watchmaker's forceps (#5), and make an incision on it using a fresh pair of autoclaved fine dissection scissors. While pulling the peritoneum, cut it along the left-right axis. Dissect out the uterus by cutting oviducts and the cervix and place it into a fresh 10-cm dish filled with 10 ml PBS. The uterus should not touch the hair or skin to maintain its sterility.
5. Cut the uterus into units with single embryos. Dissect out embryos by poking the exposed amnion using two forceps.
6. Transfer embryos into a fresh 10-cm dish filled with 20 ml PBS and remove the head, liver, heart, and any other tissues with blood. What is left looks like a jacket.
7. Gelatin-coat one 10-cm dish per embryo.

8. Collect "embryo jackets" into a 50 ml tube and wash with ten volumes of PBS three times.
9. Transfer the embryos to a 10-cm dish with 1 ml PBS covering them and mince using a sterile razor blade until a slurry-like consistency is achieved. Add 10 ml TrypLE™ Express to the dish and collect the slurry to a 50 ml tube.
10. Place the tube at 37 °C for 3 min with occasional agitation by hand. Pipette up and down the slurry ten times and allow undigested tissue fragments to settle.
11. Transfer the supernatant to a 50 ml tube with 25 ml 10 % FBS in DMEM (or LIF– medium) on ice.
12. Add 5 ml fresh TrypLE™ Express to the undigested tissue and repeat **steps 11** and **12** three more times (a total of 50 ml cell suspension).
13. Mix the cell suspension and allow any tissue fragments to settle for about 1 min.
14. Distribute the cell suspension to gelatin-coated 10-cm dishes and bring the volume of the medium to 10 ml per dish using fresh 10 % FBS in DMEM. Incubate the dishes at 37 °C, 5 % CO_2. Replace the medium next day.
15. On the following day, expand one 10-cm dish into three 10-cm dishes as passage 1 to expand a cuture (*see* Section 3.1.3). Harvest cells in the rest of 10-cm dishes. Centrifuge cells at 500 × *g* for 5 min and resuspend them in freezing medium for feeders (i.e., 10 % DMSO in the culture medium used) to cryopreserve them at passage 1 (*see* **Note 8**).
16. In 2 days, cultures at passage 1 will reach confluency (i.e., the cell–cell contact becomes very tight). Expand three 10-cm dishes into five to seven 15-cm dishes as passage 2.
17. When cultures reach confluency in 2–3 days, treat cells with 10 μg/ml Mitomycin C in culture medium for 2 h at 37 °C, 5 % CO_2 (*see* **Note 3**).
18. Remove culture supernatant with Mitomycin C and wash cells with PBS three times. Harvest and cryopreserve cells as passage 3 Mitomycin C-treated cells (three cryovials per 15-cm dish, 0.5 ml/vial). A vial of Mitomycin C-treated feeders prepared in this manner is good to plate into a 10-cm dish or a plate in a multi-well format.

*3.1.2 Serum Test (See **Note 2**)*

1. Obtain one or two samples of FBS from two to four different companies.
2. Plate 400 mESCs on each well of a 24-well plate coated with gelatin and culture them at 37 °C, 5 % CO_2.
3. Next day, feed mESCs with culture medium with LIF supplemented with 10, 15, or 30 % FBS to be tested. Change the media every 2 days and maintain the culture for 6–7 days.

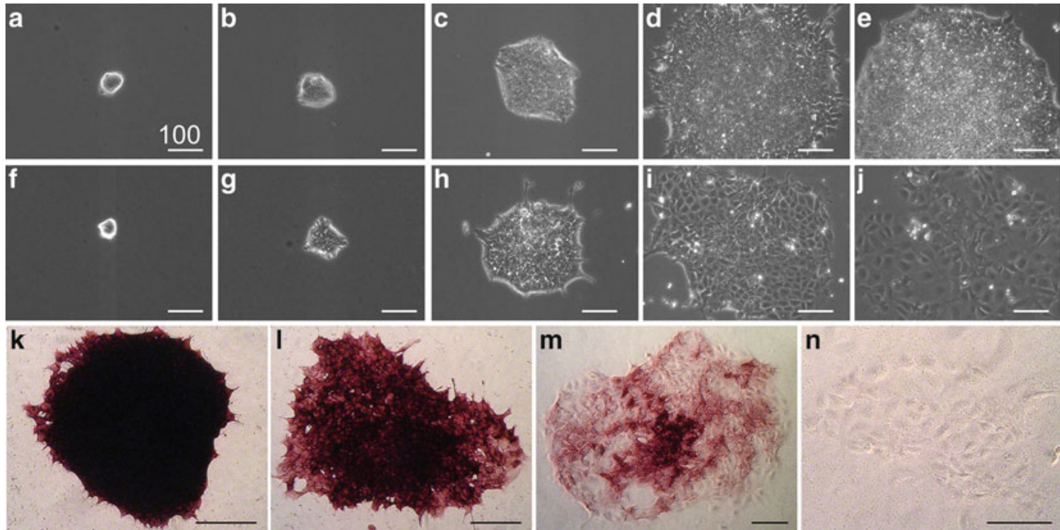

Fig. 1 Morphology of mESC colonies. Mouse ESCs were plated at 100 cells/cm^2 with complete ESC culture medium. Next day, one culture was maintained with complete ESC culture medium (**a**–**e**, **k**), whereas the other, LIF− medium (**f**–**j**, **l**–**n**). Images of representative mESC colonies were taken on 1 day (**a** and **f**), 2 days (**b** and **g**), 3 days (**c** and **h**), 4 days (**d** and **i**), and 5 days (**e**, **j**, **k**–**n**) after withdrawal of LIF. Colonies shown in **k**–**n** were stained to measure alkaline phosphatase activity. Self-renewing mESC colonies exhibit a round, packed, dome-like appearance (**a**–**e**, **k**), whereas differentiating mESC colonies show more spread, flattened morphology (**h**–**j**, **l**–**n**) with less alkaline phosphatase activity (**l**–**n**). Bars, 100 μm

4. Count the number of round, packed, dome-like colonies (Fig. 1) in each well. Alkaline phosphatase activity can be measured as well (Fig. 1; *see* Section 3.3.2). Choose the FBS lot that generates the most round, packed, dome-like colonies with high alkaline phosphatase activity even if it was used at 30 %.

3.1.3 Recovery and Expansion of Mouse Embryonic Stem Cells

1. Thaw Mitomycin C-treated feeders in warm water for a few min.
2. Add 0.5 ml 10 % FBS in DMEM (or LIF− medium) dropwise to gradually dilute the freezing medium and transfer the whole content into a 15 ml tube filled with 9 ml 10 % FBS in DMEM. Centrifuge at 500 × *g* for 5 min.
3. Aspirate supernatant, resuspend the pellet with 10 % FBS in DMEM and plate feeders on a 10-cm tissue culture dish. Incubate the culture at 37 °C, 5 % CO_2.
4. Next day, thaw mESCs similarly. Use 15 ml complete ESC culture medium (Table 2) to plate them on feeders.
5. Next day, round, packed, dome-like colonies of undifferentiated mESCs will appear (Fig. 1). Feed mESCs with fresh complete ESC culture medium every day.

6. When mESC culture reaches confluency (i.e., colonies start to touch or merge) in 2–3 days, warm TrypLE™ Express at RT. Remove old culture medium and add 1.5–2 ml 1× PBS (Table 2). Rock the plate few times by hands.
7. Aspirate PBS, add 1.5 ml TrypLE™ Express (Table 2) and incubate at RT no longer than 5 min. As trypsin digests cell surface proteins, cells start to dislodge from the bottom of the dish.
8. To neutralize TrypLE™ Express, add the same volume of complete ESC culture medium. Pipet up and down several times to make consistent cellular suspension. **Steps 6–8** are referred to as trypsinization and counted as one passage.
9. To separate mESCs from feeders, transfer the cell suspension into a 10-cm tissue culture dish (without gelatin-coating) filled with 10 ml fresh complete ESC culture medium and incubate at 37 °C, 5 % CO_2 for 40 min (*see* **Note 9**).
10. Gently pipet up and down the culture medium to rinse the bottom of the culture dish, and transfer 10 ml supernatant into a fresh, gelatin-coated 10-cm dish filled with 5 ml complete ESC culture medium.
11. Feed mESCs with fresh complete ESC culture medium every day. When the culture reaches confluency in 2–3 days, trypsinize mESCs and expand 1:5 to 1:10 into gelatin-coated 10-cm dishes (*see* **Note 10**).

*3.1.4 Detection of Mycoplasma Contamination by Nested PCR (See **Note 11**)*

1. Collect and boil 100 μl culture supernatant for 5 min. Centrifuge it at a maximum speed for 1 min.
2. Take 5 μl as a template for 50 μl reaction.
3. Assemble first PCR using MCGpF11 and MCGpR21 primers and run the reaction as follows:
   Initial denaturation at 94 °C for 1 min 30 s; 30 cycles of denaturation at 94 °C for 10 s, annealing at 55 °C for 30 s, and extension at 68 °C for 1 min; final extension at 68 °C for 7 min 30 s and store at RT.
4. Take 1 μl of the first PCR as a template for 50 μl reaction.
5. Assemble second PCR using either R16-2 or MCGpF2 as a forward primer, and MCGpR21 as a reverse primer (i.e., two second reactions per sample), and run them as above.
6. Run all kinds of PCR products on 1 % agarose at 10 V/cm.

3.2 Delivery of a Transgene into Mouse Embryonic Stem Cells

While it takes several days to test the pluripotency of mESCs using assays described in Section 3.3, typical plasmids that harbor a transgene expression cassette do not replicate in mESCs and will be eventually lost due to the lack of a proper replication origin (Ori), unless stably integrated into the genome. Thus, the

following protocols focus on generating such stable mESC lines through simultaneous introduction of another cassette for antibiotic resistant gene expression (either built in the same plasmid or provided by another plasmid, i.e., co-transfection of two kinds of plasmids). These approaches randomly introduce multiple copies of a transgene into single mESCs. Thus, to examine the role that a transgene of interest plays in mESCs and eliminate the effect of transgene insertion per se, five to eight mESC clones need to be isolated and assayed independently as biological replicates.

3.2.1 Preparation of Plasmid DNA

1. Purify plasmid DNA from *E. coli* using a commercial kit (e.g., HiSpeed Plasmid Maxi kit from Qiagen) (*see* **Note 12**).
2. Determine the concentration of purified DNA and use 20 μg (i.e., double the amount needed for delivery) for restriction enzyme digestion.
3. Set up a reaction using 30–40 units of a restriction enzyme to linearize plasmid DNA and incubate it at 37 °C overnight to achieve complete digestion.
4. Add 1/10 volumes of 3 M sodium acetate pH 5.2 and 2 volumes of 100 % EtOH, mix well by inverting the tube several times, and leave it at −20 °C overnight.
5. Centrifuge precipitated DNA at a maximum speed for 30 min at RT.
6. Discard the supernatant and rinse the pellet with 70 % EtOH by inverting the tube several times. Centrifuge rinsed DNA at a maximum speed for 5 min at RT.
7. Resuspend DNA into 11 μl pure H_2O. Take 0.5 μl to measure its concentration, and another 0.5 μl to run it on 0.8 % agarose gel at 10 V/cm and confirm that plasmid DNA is linearized (*see* **Note 13**).

3.2.2 Electroporation

1. Harvest mESCs by trypsinization. Resuspend 1–20 million mESCs in 750 μl PBS and transfer the suspension into an ice-chilled 4-mm cuvette.
2. Add 10 μg linearized plasmid DNA into the cuvette and adjust the total volume to 800 μl. Leave the cuvette on ice for 5 min.
3. Wipe the side of the cuvette and apply an electric field to it as follows: 250 V, 500 μF for Bio-Rad Gene Pulser; 325 V, 99 μs/pulse × 12 pulses for BTX ECM 200. Leave the cuvette on ice for 5 min (*see* **Note 14**).
4. Add 1 ml warm complete ESC culture medium in the cuvette using a 6 ml transfer pipette, mix well and transfer the whole content into a gelatin-coated 10-cm dish filled with 15 ml complete ESC culture medium. Incubate the culture at 37 °C, 5 % CO_2. Next day, start selection with a drug (*see* **Note 4**).

3.2.3 Nucleofection

1. Harvest mESCs by trypsinization. Resuspend two to five million ESCs in 100 μl complete Nucleofector solution provided by the kit.
2. Mix cells with 1–10 μg linearized plasmid DNA. The volume of plasmid DNA should be less than 10 μl. There is no need to adjust the total volume to 100 μl but should be less than 110 μl.
3. Transfer the cell–DNA mix into an ice-chilled 4-mm cuvette and leave it on ice for 5 min.
4. Wipe the side of the cuvette and apply it to the nucleofector. Start the predefined program (*see* **Note 15**). Keep the cuvette on ice for 5 min.
5. Add 1 ml warm complete ESC culture medium in the cuvette using a transfer pipette provided by the kit, mix well and transfer the whole content into a gelatin-coated 10-cm dish filled with 15 ml complete ESC culture medium. Incubate the culture at 37 °C, 5 % CO_2. Next day, start selection with a drug.

3.2.4 Lipofection

1. A day before transfection, plate 1×10^6 mESCs in 2 ml complete ESC culture medium per gelatin-coated well of a 6-well plate.
2. Next day the culture reaches about 70 % confluency. Feed cells with 2 ml fresh complete ESC culture medium.
3. Allow FuGENE® HD, linearized plasmid DNA and PBS to be adjusted at RT.
4. Dilute 2 μg linearized plasmid DNA with 1× PBS and adjust the volume to 100 μl.
5. Mix 9 μl FuGENE® HD with DNA in PBS vigorously by pipetting. Incubate it for 15 min at RT.
6. Add the DNA-FuGENE® HD complex into the culture drop wise, swirl the plate to mix the DNA-FuGENE® HD complex with the medium and leave the culture at 37 °C, 5 % CO_2 overnight (*see* **Note 16**). Next day, start selection with a drug.

3.2.5 Retrovirus/ Lentivirus

1. Submit a protocol to the IBC and obtain approval before conducting the following experiment due to the use of human cells (i.e., Phoenix™ Eco or HEK293T cells).
2. Produce active viruses as follows:
   (a) Culture a packaging cell line (e.g., Phoenix™ cells for ecotropic retrovirus and HEK293T cells for lentivirus) with 10 % FBS in DMEM with 1× GlutaMAX™ and penicillin–streptomycin at 37 °C, 5 % CO_2 (*see* Section 2.1 and **Note 17**).

(b) A day before transfection of proviral vectors, plate 2–3 × 10^6 cells in a 10-cm dish.

(c) Next day, mix either 30–40 μg supercoiled proviral vector for retrovirus, or 10 μg proviral vector, 9 μg pLP1, 4 μg pLP2, and 6 μg pLP/VSVG for lentivirus, in 438 μl H_2O, with 62 μl of 2 M $CaCl_2$ in a 14 ml round-bottom tube per transfection.

(d) While vortexing the tube vigorously, add 500 μl 2× HeBS drop-wise with a P1000 pipetman.

(e) Add the DNA–$CaCl_2$ mixture into the culture of packaging cells drop-wise while swirling the dish gently. Precipitates of DNA should be visible under a microscope. Incubate the culture at 37 °C, 5 % CO_2 overnight.

(f) Next day in the morning, discard about a half of the culture supernatant, feed cells with 5 ml fresh medium and culture them at 37 °C, 5 % CO_2. For retrovirus, collect culture supernatant in 2–3 days. For lentivirus, collect culture supernatant every day for 2–3 days. Store collected culture supernatant at 4 °C (*see* **Note 18**).

(g) Filter-sterilize collected culture supernatant to remove cellular debris. For retrovirus, make aliquots (0.4 ml each). For lentivirus, ultracentrifuge the culture supernatant at 25,000 × *g* for 2 h at 4 °C, resuspend the pellet into 0.5–1 ml PBS and make aliquots (100 μl each). Store aliquots at −80 °C and do not repeat freezing and thawing them.

3. Infect viruses to mESCs as follows:

(a) A day before infection, plate 3 × 10^6 mESCs in a gelatin-coated 10-cm dish with complete ESC culture medium.

(b) Thaw the virus stock at RT and dilute it with 4 ml complete ESC culture medium with 4 μg/ml polybrene (*see* **Note 19**).

(c) Aspirate old culture supernatant, add the medium with diluted virus to mESCs and incubate them at 37 °C, 5 % CO_2 on a rocker platform for 3–5 h.

(d) Add 6 ml more complete ESC culture medium with 4 μg/ml polybrene and further incubate the culture overnight (no rocking). Next day, start drug selection of infected mESCs. Up to about a 100 drug-resistant colonies will appear in 10 days for retrovirus, fivefold less for lentivirus.

3.2.6 Colony Picking and Expansion in a 96-Well Format

1. Disinfect a dissection microscope using 70 % EtOH and put it in the biosafety cabinet.
2. Dispense 25 μl TrypLE™ Express into each well of a v-bottom 96-well plate and keep the plate on ice in the biosafety cabinet.

3. Aspirate old medium from the culture of drug-resistant colonies and add 10 ml PBS into the culture.
4. Identify a colony with a round, packed, dome-like shape of a similar size under the microscope and pick it up using a P20 pipetman. Set the scale to 5–10 μl to minimize carrying PBS to TrypLE™ Express.
5. Transfer the colony into a v-bottom well with TrypLE™ Express and repeat **steps 4** and **5**. Keep isolated colonies on ice until a number of colonies are collected (*see* **Note 20**).
6. Incubate isolated colonies at 37 °C, 5 % CO_2 for 5 min to dissociate them.
7. Coat each well of a fresh 96-well plate with 0.1 % gelatin in PBS. After aspirating the gelatin solution, fill each well with 200 μl warm complete ESC culture medium.
8. Take 25 μl culture medium from each well of the fresh 96-well plate and add it to the corresponding well of the v-bottom plate to inactivate TrypLE™ Express. Pipet up and down several times.
9. Transfer each whole cell suspension in the v-bottom plate into the corresponding well of the fresh 96-well plate with complete ESC culture medium, pipet up and down several times and incubate at 37 °C, 5 % CO_2 (count as passage 0).
10. Next day feed cells with 200 μl fresh culture medium.
11. When the cultures reach confluency, trypsinize them using 100 μl each TrypLE™ Express. Add 100 μl complete ESC culture medium to neutralize TrypLE™ Express. To maintain ongoing culture, plate 20 μl cell suspension into each fresh well coated with gelatin (plating at 1:10). To make a frozen stock, take 60 μl each cell suspension and mix it with 60 μl each 2× freezing medium in an 8-tube PCR strip column-wise. Overlay a few drops of H_2O-washed mineral oil, close lids, wrap with thick paper towel and store at −80 °C. With this recipe, frozen stock can be made in triplicate.

3.3 Measurement of Mouse Embryonic Stem Cell Pluripotency

Pluripotency of mESCs can be evaluated by their abilities to differentiate in vitro and in vivo (i.e., formation of chimeric mice), to form embryoid bodies (EBs) and to develop into a teratoma (i.e., a tumor with specialized cells derived from three germ layers), high alkaline phosphatase activity, and expression of markers such as Oct3/4, Nanog, Sox2, and SSEA1.

3.3.1 Induction of Random Cell Differentiation

1. A day before withdrawal of LIF, harvest and plate mESCs at 100 cells/cm^2 on gelatin-coated dishes/wells with fresh complete ESC culture medium and maintain them at 37 °C, 5 % CO_2 (*see* **Note 21**).

2. Next day, remove old medium, wash cultures once with PBS and feed one set of mESC cultures with fresh complete ESC culture medium, and the other set using LIF− medium. This is counted as Day 0. Feed cells with fresh media every day.
3. At day 3, the appearance of colonies cultured under LIF− conditions becomes distinct from that of colonies under LIF+ conditions (Fig. 1).
4. At day 5, culture exhibits significant random differentiation under LIF− conditions (Fig. 1). Cultures can be fixed with alcoholic solution or 4 % paraformaldehyde at this point for further characterization of marker expression (*see* Section 3.3.3).

3.3.2 The Alkaline Phosphatase Assay

1. Remove old culture medium and rinse cultures with 1× PBS. Fix mESC cultures with ice-cold alcoholic solution (e.g., Methanol/Ethanol: DMSO = 4:1) for 10 min on ice.
2. Wash fixed cells with PBS three times and add 2 ml substrate for alkaline phosphatase reactions.
3. Incubate at RT until desired color develops (15 min to 1 h; Fig. 1; *see* **Note 22**).

3.3.3 Immunofluorescence Microscopy

1. Culture cells on 3.5-cm dishes or multi-well plates (*see* **Note 23**).
2. Remove old culture medium and rinse cultures with 1× PBS. Fix mESCs with 4 % PFA in 1× PBS at RT for 10 min. Refer to the volume of PBS listed in Table 2.
3. Wash fixative with PBS three times. Refer to the volume of medium listed in Table 2.
4. Block samples with PBSMT at RT for 0.5–1 h. Refer to the volume of medium listed in Table 2.
5. Incubate blocked samples with primary antibodies diluted in PBSMT at 4 °C overnight (*see* **Note 24**). Shaking is not necessary. Refer to the volume of TrypLE™ Express listed in Table 2.
6. Prechill PBSMT at 4 °C for at least 1 h. Wash samples twice with PBSMT for 10 min each at 4 °C. Shaking is not necessary. Refer to the volume of medium listed in Table 2.
7. Wash samples three times with PBSMT for 10 min each at RT. Shaking is not necessary. Refer to the volume of medium listed in Table 2.
8. Incubate samples with fluorophore-conjugated secondary antibodies diluted in PBSMT 1:400 at RT for 2 h or 4 °C overnight. Refer to the volume of TrypLE™ Express listed in Table 2. Cover samples with aluminum foil for the rest of the steps.
9. Wash samples as **steps 6** and **7** if incubation is carried out at 4 °C. Otherwise, wash samples five times with PBSMT for 10 min each at RT. Shaking is not necessary. Refer to the volume of medium listed in Table 2.

10. Before microscopic observation, incubate samples with 0.5 μg/ml DAPI in 1× PBS at RT for 20 min (*see* **Note 25**).

3.3.4 Hanging Drop Culture Followed by Induction of Cardiac Myocytes

1. Harvest and resuspend mESCs at 5×10^4 cells in 2.5 ml LIF− medium.
2. Make 50–80 drops (25 μl each) on the lid of a petri dish and record the number of drops made.
3. Add 10 ml PBS in the petri dish, flip the lid with drops using one hand and put it back on the dish with PBS. Incubate it at 37 °C, 5 % CO_2 for 3–4 days. A cellular aggregate called embryoid body (EB) is formed in each drop if mESCs are pluripotent.
4. Add 1 ml LIF− medium into a 0.1 % gelatin-coated well of a 24-well plate. Transfer 30–40 EBs into the well. Increase the number of wells according to the number of hanging drops made.
5. Count EBs and compare the number of EBs with the number of hanging drops. If mESCs maintain pluripotency, almost 100 % of the hanging drops will form EBs. If not, then the pluripotency of mESCs may have been compromised. When mESCs were maintained with LIF− medium for 4–6 days prior to this EB formation assay, about 60 % of drops produced EBs.
6. Incubate EBs at 37 °C, 5 % CO_2 and feed them every 2 days. In 7–14 days beating foci of cardiac myocytes will appear if mESCs used to make EBs are pluripotent. Beating foci will be pronounced a few hours after they are fed with fresh medium.

3.3.5 Formation and Validation of a Teratoma

1. Prepare $1–2 \times 10^6$ mESCs in 25 μl PBS per transplantation.
2. Mix the cell suspension with the same volume of 0.3 mg/ml type IA collagen.
3. Load the cell–collagen mix into a tuberculin syringe with a 26 G needle and inject it subcutaneously into the back of an immunocompromised mouse (*see* **Note 7**).
4. A tumor becomes palpable 3 weeks after transplantation. Seek for a veterinary advice to determine the end point of the host mouse. Typically these mice reach the end point 6 weeks after transplantation.
5. Dissect out and weigh the tumor and measure its longest axis for comparisons.
6. Cut the tumor into smaller pieces (e.g., $1 \times 1 \times 1$ cm^3) on a 6-cm dish filled with PBS.
7. Collect the trimmed tumor sample into a 50 ml tube and rinse with ten volumes of PBS three times or until less blood cells are observed. Fix the tumor sample with ten volumes of 4 % PFA in 1× PBS at 4 °C overnight.

8. Remove the fixative, wash the tumor sample with 5–10 ml PBS three times, and dehydrate it with ten volumes of 70 % EtOH.
9. Replace 70 % EtOH with 80 % EtOH to start the EtOH–Histo-Clear series followed by embedding into Paraplast Plus®. Or replace 70 % EtOH with fresh 70 % EtOH for indefinite storage at RT.
10. Prepare 8 μm-thick paraffin sections on a glass slide. Deparaffinize the sections by the Histo-Clear–EtOH series.
11. Place the slides in Tupperware and submerge them in 10 mM sodium citrate buffer pH 6.0. Microwave them at level 5 for 5 min three times using a 1,250 W model.
12. When the slides cool down, proceed to immunofluorescence microscopy as described in Section 3.3.3. Mount the slides using nonfluorescent glycerol:PBS = 9:1 or glycerol–gelatin (*see* **Notes 25** and **26**).
13. Prepare another set of slides with sections cut from the same tissue block.
14. After deparaffinizing the sections, stain them with Alcian Blue, hematoxylin, and eosin sequentially. Dehydrate sections by the EtOH–Histo-Clear series and mount using Permount (*see* **Note 26**).

4 Notes

1. Feeders provide mESC culture with the differentiation inhibitory factor, leukemia inhibitory factor (1, 2) and soft substrates (9). Thawing and plating mESCs on feeders is a standard method to maintain good culture of mESCs. However, for assays, feeder-free or chemically defined serum-free cultures are suited (10–12). For this purpose, R1 mESCs can be thawed on type IA collagen (*see* Section 2.3)-coated dishes with 3 μM CHIR99021 supplemented in complete ESC culture medium, and expanded onto gelatin-coated dishes using the culture medium without significant induction of cell differentiation.
2. Use of an animal serum-based culture medium is a standard method to maintain mESCs. However, animal serum contributes to producing heterogeneous cell populations (e.g., (9, 13, 14) and references cited in (15)), because FBS may contain factors that provide a negative impact on the self-renewal of mESCs. Even though the FBS product is claimed to be qualified for mESC culture, quality of FBS significantly varies among different lots and it is recommended to test its performance. If FBS negatively regulates mESC self-renewal, a higher percentage of FBS in a culture medium will result in decrease of the number of round, packed, dome-like colonies (Fig. 1). Serum-free cultures have

been established and provide a relatively homogeneous cell population (10, 12). Although a serum-replacement is commercially available, it may exhibit lot-to-lot variation.

3. Mitomycin C is a hazardous material. Consult the IBC for its proper disposal. However, Mitomycin C can be effectively inactivated by bleach (16).
4. For R1 and W4 (17) mESCs, antibiotics can be used at the following concentration: G418, 250 μg/ml; Puromycin, 2 μg/ml; Blasticidin, 2.5 μg/ml; Zeosin, 400 μg/ml. Blasticidin in a liquid form is quite unstable at a neutral pH and reduces its activity in a couple of weeks even when stored at 4 °C. Significant reduction of its activity occurs after two cycles of freezing and thawing or longer storage at 4 °C. Thus, it is advised to increase its dose if cell death is not observed a few days after drug selection is started, or use a freshly prepared blasticidin solution. Under conditions described in Sections 3.2.2–3.2.5, typically a couple of hundreds of drug-resistant colonies will appear in 10 days. However, the number of drug resistant colonies is dependent on the purity, amount, and length of DNA. Particularly, the efficiency of liposome-based DNA delivery significantly drops when long DNA is used (18).
5. Several lipofection reagents are commercially available, whereas it is expected that a transfection reagent per se will affect gene expression. The effect of FuGENE® HD was two- to sevenfold less than that of another reagent according to the comparison of the number of genes whose expression levels were altered after transfection (19, 20).
6. Not all of the type IA collagen products are suited for this application. Cellmatrix Type I-A gives consistent results.
7. Submit a protocol to the institutional animal care and use committee (IACUC) and obtain approval before conducting the work involving the use of mice. Eight-week-old or older mice can be used for natural mating and the teratoma formation assay. For natural mating, two female mice can be left with a male mouse for a few days until a vaginal plug is noticed in the following morning. Typically a pregnant female C57BL6 or CD1/ICR mouse carries 10–13 embryos. For the teratoma formation assay, an immunocompromised mouse can accommodate four separate transplantations, two each on the right or left side. Due to a human error associated with injection, it is advised that several transplantations per cell type/line are made. Maintaining a small number of breeding colonies of immunocompromised animals may not be effective to produce an enough number of animals to perform the teratoma formation assay with replicates.

8. Use of a cryogenic freezing container with isopropanol (e.g., Nalgene Mr. Frosty™ Freezing Container) helps slowly freeze cells. A day or 2 days after placing the cells at −80 °C, it is recommended to store cells at −196 °C (i.e., in a liquid nitrogen dewar), although mESCs and feeders can be stored at −80 °C for several years.
9. Because feeders supply LIF to the culture of mESCs, feeders need to be removed before assays to test the pluripotency of mESCs are conducted. Feeders are larger than mESCs, such that they reach the bottom of the dish and start to spread faster than mESCs. It takes about 40 min for feeders to spread at 37 °C, 5 % CO_2. After one or two times of passaging in this manner, almost the entire culture is filled with mESCs.
10. Under steady and optimum conditions, mESC culture will reach confluency every other day. If mESC colonies are sparsely distributed and exhibit slower growth, they can be trypsinized to stimulate their growth. Discard culture when passaged onto gelatin-coated dishes 20 times under these conditions and start using freshly thawed mESCs. It is advised to prepare multiple cryovials of mESCs at early passages.
11. Culture of mESCs should be free of mycoplasma contamination, otherwise the pluripotency of mESCs will be compromised (i.e., mESCs contaminated with mycoplasma will not transmit to germ-line). Use of 30 μg/ml Kanamycin sulfate may prevent cultures from contaminating with mycoplasma. If mESC culture is contaminated with mycoplasma, 100–660 bp products will be identified by the method described in Section 3.1.4. The size of a PCR product varies depending on the strain of mycoplasma (21, 22). Additionally, mycoplasma contamination can be detected by Hoechst 33258, which will show fluorescence in the cytoplasm.
12. Purity of plasmid DNA significantly affects the transfection efficiency, and commercial kits simply provide the purity. To make sure that a cassette that forces the expression of a transgene is integrated in the host cell genome in its original form, the plasmid DNA encoding the cassette needs to be linearized. However, it is highly likely that ends of the plasmid DNA will be lost (i.e., chewed back) during the delivery-integration process. Therefore, linearized plasmid DNA must have nonessential buffer sequences from several hundred bps to a couple of kbs at the ends.
13. It is common to lose about a half of plasmid DNA used by this procedure. One to two microgram linear acrylamide can be added to enzyme-digested DNA to increase the recovery during EtOH precipitation.

14. Good electroporation conditions will likely kill about 50 % of electroporated cells (23).
15. The program A-023 works well with R1 mESCs. Often an error message “Weak” appears even though a fresh cuvette and a nucleofection solution for mESCs are used. However, most likely DNA is delivered inside the cells.
16. The scale of culture and the amount of plasmid DNA and FuGENE® HD used can be proportionally increased/decreased according to the surface area of the culture (*see* Table 2). After adding the DNA-FuGENE® HD complex into culture, pipetting to mix the complex with the medium does not help with increasing the transfection efficiency and should be avoided. Overnight incubation with the complex is necessary to achieve the maximum transfection efficiency.
17. Phoenix™ cells are derived from HEK293T cells (3, 4). They will reach 70–80 % confluency in every 2 days under steady and optimum conditions. Passage them at 1:5 by trypsinization. Alternatively, routine passaging can be done by mechanical separation, because HEK293T cells very loosely attach the bottom of a culture dish. LIF− medium cannot be used. Perhaps sodium pyruvate, NEAA, and/or 2-ME negatively regulate the growth of HEK293T cells. Every once in a few months of culture, it is recommended to select Phoenix™ cells with 300 μg/ml Hygromycin B and 1 μg/ml Diphtheria Toxin for 1 week to maintain their high-titer virus production. Then, a large number of stocks at early passages should be prepared.
18. Viruses produced in these methods are unstable. Culturing transfected cells at 30 °C may help to reduce degradation of viruses, while virus production becomes slow. Also, keeping culture supernatant at lower temperature will help retain a high virus titer. Cell lines stably producing retroviruses can be established with Phoenix™ cells (24). The lines with a high virus titer can be identified by dot blotting using part of the transgene expression cassette as a probe. Retroviruses produced with Phoenix™ Eco cells are ecotropic (i.e., infect only mouse cells) and do not infect Phoenix™ cells, whereas lentiviruses will infect HEK293T cells as produced. Co-transfection of a proviral retrovirus vector with pLP/VSVG into Phoenix™ Eco cells will make ultracentrifugation of retroviruses possible to concentrate them, but they become amphotropic (i.e., infect human cells, too).
19. Polybrene enhances the single infection of virus. With this protocol, less than 1 % of drug-resistant clones had multiple integrations of viruses detected by southern blotting, although exact multiplicity of infection (MOI) is unknown.

20. The expression level of a transgene depends on the copy number of the vector and activity of the neighborhood gene transcription units, once the expression vector is stably integrated into the genome (25). Therefore, expression levels of a transgene in established mESC lines need to be validated by total RNA extraction followed by either Northern blotting or quantitative RT-PCR. To alleviate this process, a new expression vector has been developed (13). This vector enables us to isolate stable clones with a strong expression level of a transgene by the CAG promoter (26) combined with a translational enhancer (25, 27) and transcriptional enhancer (28). In addition, DsRedT3 (29, 30) is linked with the immediate downstream of the transgene by the self-cleaving peptide T2A (31). Therefore, red fluorescence helps us isolate clonal mESCs that exhibit a high expression level of a transgene (13). Typically, using an object marker available through Nikon, we mark drug-resistant colonies that exhibit red fluorescence a day or 2 days before colony picking and pick 16 clones per transgene. About a half of the clones show weak red fluorescence once expanded, but the rest maintains high red fluorescence. In this manner, data can be statistically tested with high statistical power.
21. Mouse ESC self-renewal depends on LIF as well as autocrine factors (32–35). Thus, perturbation of gene expression and/or random insertion of expression vectors may result in suppression of the self-renewal at this seeding density. If this is observed, the seeding density needs to be increased.
22. This reaction is completely inhibited by 0.3–5 mM levamisole. Thus, it is likely that mESCs express tissue nonspecific alkaline phosphatase as primordial germ cells and embryonic carcinoma cells do, but do not express embryonic alkaline phosphatase (36–39).
23. Glass bottom dishes available through MatTek Corp. will provide clearer microscopic images. However, we found that R1 mESCs do not grow well on glass surface even though it is treated with 0.1 % gelatin or 15 mg/ml type I-A collagen possibly due to the stiffness of the substrate (9). Thus, we routinely image mESCs cultured on plastic dishes. Substrates available from Ibidi may offer a better alternative (http://ibidi.com).
24. The dilution factor is dependent on the titer of the antibody. However, as a starting point, a purified antibody can be diluted at 1–2 μg/ml because that is the typical concentration of a monoclonal antibody secreted into culture supernatant. If the concentration of the antibody used is too high, this will result in nonspecific binding and high background. Diluted

antibodies can be reused for a few times if stored at 4 °C with 0.1 % NaN_3. To increase signal-to-noise ratio, Image-it® FX signal enhancer (Life Technologies I36933) can be used as an alternative to PBSMT (13). Use a humidified chamber to prevent diluted antibodies from drying. Cover samples with aluminum foil for the rest of the steps if the primary antibody is conjugated with a fluorophore.

25. Stained samples can be stored in 0.5 μg/ml DAPI in PBS. Alternatively, nonfluorescent glycerol:PBS = 9:1 or glycerol gelatin (Sigma GG1) are suited to mount samples. Either way samples should be stored at 4 °C and good for a week, or the fluorescence will become decreased because of the diffusion of antibodies. Although significant photobleaching of fluorescence may become an issue, several chemicals are known to retard photobleaching during fluorescence microscopy (40–43) and anti-fading agents are commercially available (e.g., ProLong Gold® Antifade Mountant, Life Technologies P10144; 13).
26. Nestin, α-smooth muscle actin, and α-fetoprotein are widely used markers of ectoderm, mesoderm, and endoderm, respectively (9, 44). On the other hand, specialized cells derived from these germ layers can be clearly distinguished by histological staining (9, 11, 44). For example, epithelial precursors called keratin pearls will exhibit eosin-stained (i.e., red) keratin surrounded by hematoxylin-stained (i.e., purple) epithelium. Neural precursors will be densely stained purple with hematoxylin and exhibit tube/rosette-like structures. Due to the deposition of chondroitin sulfate, cartilages will be stained blue with Alcian Blue. Striated muscles will be stained red with eosin and recognized by the appearance of sarcomere repeats at higher magnification. Ciliated epithelia are found in the intestine and typically identified by a layer of cells with hematoxylin-stained nuclei localized on the basal side and the eosin-stained cytoplasm on the apical side facing the lumen.

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Generation of a Knockout Mouse Embryonic Stem Cell Line Using a Paired CRISPR/Cas9 Genome Engineering Tool

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Abstract

CRISPR/Cas9, originally discovered as a bacterial immune system, has recently been engineered into the latest tool to successfully introduce site-specific mutations in a variety of different organisms. Composed only of the Cas9 protein as well as one engineered guide RNA for its functionality, this system is much less complex in its setup and easier to handle than other guided nucleases such as Zinc-finger nucleases or TALENs.

Here, we describe the simultaneous transfection of two paired CRISPR sgRNAs-Cas9 plasmids, in mouse embryonic stem cells (mESCs), resulting in the knockout of the selected target gene. Together with a four primer-evaluation system, it poses an efficient way to generate new independent knockout mouse embryonic stem cell lines.

Keywords: CRISPR/Cas9, CRISPR-Cas9 paired design, mESCs: mouse embryonic stem cells, (Homozygous) knockout, Genome engineering

1 Introduction

Mouse embryonic stem cells (mESCs) can be derived from the inner cell mass of the blastocyst at 3.5 days post-coïtum (dpc). In appropriate culture conditions (e.g., BMP/LIF), they retain their pluripotent state and are able to proliferate indefinitely. Three proteins form the core factors essential for the maintenance of stem cell pluripotency, namely Oct4 (Pou5f1), Sox2, and Nanog (1, 2). Their complex network of interaction with each other or with additional pluripotency factors and transcription factors provides a fragile balance between stemness and the possibility for cell fate specification via differentiation (3). Stem cells harbor an immense developmental potential as they can differentiate into three germ layers and eventually all tissue types. From a medical point of view, these abilities of stem cells, together with technical progress, open up new possibilities for the investigation of genetic diseases. A better understanding of stem cells in biology is a key step

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in the development of personalized medicine (4). Several stem cell lines have been established from embryonic and adult tissues, but the mESCs are a comparatively easy to handle model suited to address very diverse questions in biology and medical science.

In the past years, a number of genome engineering tools such as Zinc-finger nucleases and TALENs have been used to introduce targeted modifications in the genome. Lately, CRISPR/Cas9 has emerged as a new tool and proven to be an easy and cost-effective application (5).

Although interspersed repeats were first mentioned in 1987 (6), the term "Clustered Regularly Interspersed Short Palindromic Repeats," shortly CRISPR, emerged only in 2002 when Jansen and Mojica used the term to describe genomic loci, containing repeated DNA sequences interspersed by nonrepetitive elements in microbes (7). Recently, CRISPRs have been more closely and intensively investigated and were identified as a defence system used by bacteria against foreign DNA, derived from pathogens such as viruses (8). Thanks to a better understanding of the system, scientists have been able to adapt the CRISPR/Cas9 defence system for genome engineering in a wide range of organisms. Many publications proved this system to be an effective and efficient tool to generate targeted genome modifications (9, 10). Among the three types of the CRISPR system found in bacteria, type II CRISPR/Cas9 from *Streptococcus pyogenes* is the most suitable for genome editing and therefore also most investigated. It consists of a CRISPR-associated protein 9 (Cas9) and two RNAs—CRISPR RNA (crRNA) and trans-acting CRISPR RNA (tracrRNA)—which are partially complementary to each other and form a duplex. The crRNA and tracrRNA can be fused to form a single guide RNA (sgRNA), simplifying the use of the CRISPR/Cas9 as a genome engineering tool (11, 12). The system gains its specificity from the sgRNA, which is complementary to a sequence in the target genome, and the so-called protospacer adjacent motif (PAM), a defined 3 nt sequence at the 3′ end of the target DNA sequence (13–15). The sgRNA guides the Cas9 protein to its site of action where it recognizes the PAM and cleaves the DNA using its two catalytic subunits HNH and RuvC, inducing a double-strand break (DSB) (16). Then, DSBs caused by CRISPR/Cas9 will be repaired by the cell either using nonhomologous end joining (NHEJ), resulting in small insertions/deletions at the ligation site, or by homology-directed repair (HDR), requiring a template for perfect repair. Generally, NHEJ is preferred over HDR in case of CRISPR/Cas9 DSBs but the repair mode can be guided toward HDR by the use of a modified Cas9 protein, which only nicks the DNA instead of cleaving it (10). Recent modifications of the Cas9 protein allow a broader employment of the CRISPR/Cas9 system going beyond genome editing. The use of catalytically inactive

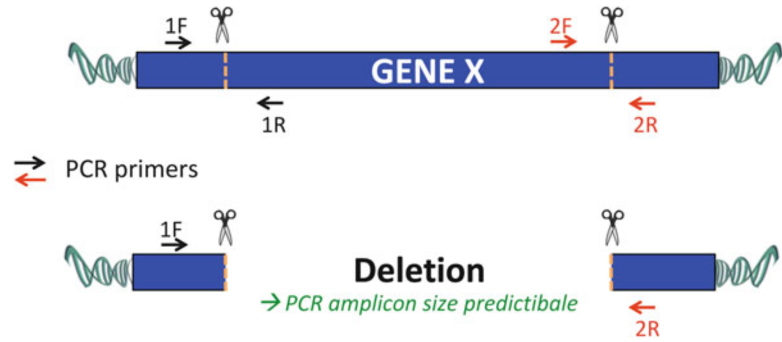

Fig. 1 Overview of the paired CRISPR/Cas9 strategy. In this protocol, specific CRISPR/Cas9 constructs are designed and used in pairs in order to induce two cuts in the genomic DNA involving the generation of a deletion. Then specific PCR primers on both side of each CRISPR/Cas9 target site are designed. By combining the forward primer of the upstream CRISPR/Cas9 target site (called 1F) and the reverse primer of the downstream CRISPR/Cas9 target site (called 2R), it is possible to distinguish the "wild type" amplicon, resulting from the amplification of the wild type allele, from the "deleted" amplicon, shorter, resulting from the amplification of the deleted allele. This PCR allows the assessment of the efficiency of CRISPR/Cas9 pairs as well as the screen of the independent clones by distinguishing wild type, heterozygous and homozygous mutant clones from each other

Cas9 (dCas9) fused to activator or inhibitor attracting molecules allows specific endogenous gene regulation posing an alternative to conventional RNA interference (RNAi) (17, 18). Fused to fluorescent molecules, dCas9 can also serve as an imaging tool, visualizing specific loci on the genome (19). Additionally, O'Connell and colleagues found a way to guide Cas9 also to ssRNA, opening up new possibilities to study RNA function (20). To date several online tools, such as e-CRISP (21) and Feng Zhang Lab's target finder (22), have been published, simplifying the design of sgRNAs and prediction of possible off-target effects (23–25). These tools allow rapid and efficient generation of CRISPR target sites resulting in genome scale screening libraries (26).

In our studies, we employed a combination of two CRISPR sgRNAs targeting a selected gene together with an easily handled four primer-evaluation system (*see* Fig. 1). By cutting the target gene twice in close proximity, a piece of the DNA is cut out, creating a deletion in the gene of interest, rendering it nonfunctional (27). Even though deletions up to 1 Mb can be achieved (28), careful positioning of the two sgRNA target sites and the verification primers allow easy detection of successful deletions of around 2 kb by PCR as well as distinction between homo- and heterozygous mutants. The specific steps of this protocol are described in the following sections.

2 Materials

2.1 Design of Paired CRISPR/Cas9 sgRNAs and PCR Primers

1. Genomic DNA sequences (*see* **Note 1**).
2. sgRNA CRISPR design software (*see* **Note 2**).
3. PCR primer design software (*see* **Note 3**).

2.2 Construction of Specific CRISPR/Cas9 Vectors Using the pSpCas9 (BB)-2A-GFP Plasmid

1. pSpCas9(BB)-2A-GFP (Addgene plasmid ID: 48138), also called pX458 (29).
2. BpiI (BbsI) enzyme, 10 U/μL and its related buffer G (Thermo Scientific).
3. Agarose powder (Invitrogen).
4. Ethidium Bromide Solution 10 mg/mL (Bio-Rad).
5. 6× Loading dye (Thermo Scientific).
6. 2-Log DNA Ladder (0.1–10.0 kb) (New England BioLabs).
7. Electrophoresis DNA power supply and horizontal electrophoresis cell (Bio-Rad).
8. UV revelation device for DNA electrophoresis gel.
9. QIAquick® Gel extraction Kit (Qiagen).
10. T4 DNA ligase and its associated 10× T4 DNA Ligase Buffer (Thermo Scientific).
11. One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen).
12. Lysogeny Broth (LB) medium (BD).
13. Autoclaved water.
14. Ampicillin (Applichem). Prepare 100 mg/mL stock solution by diluting 100 mg of Ampicillin powder into 1 mL of autoclaved water. This stock solution must be stored at −20 °C.
15. Ampicillin agar plates (Ampicillin at 0.1 mg/mL final concentration).
16. GeneJET Plasmid Miniprep Kit (Thermo Scientific).
17. Pure Link™ HiPure Plasmid Filter Maxiprep Kit (Invitrogen).

2.3 Efficacy Assay of Paired Designed CRISPR/Cas9 Constructs

1. mESC proliferative medium: Dulbecco's Modified Eagle Media (DMEM) (Invitrogen) containing 15 % of a special selected batch of fetal bovine serum (FBS; Life Technologies) tested for optimal growth of mESCs, 1,000 U/mL of LIF (Millipore), 0.1 mM of 2-β-mercapto-ethanol (Life Technologies), 0.05 mg/mL of streptomycin, and 50 U/mL of penicillin (Sigma).
2. mESC differentiation medium: DMEM (Invitrogen) containing 10 % of FBS (Life Technologies), 0.1 mM of 2-β-mercapto-ethanol (Life Technologies), 0.05 mg/mL of streptomycin and 50 U/mL of penicillin (Sigma). This medium is LIF-free.

3. 0.2 % gelatin solution and gelatin-coated flask/plate. Prepare 500 mL of 0.2 % gelatin solution: dissolve 1 g of gelatin from porcine skin (Sigma) with 500 mL of autoclaved water, mix well and then autoclave the solution. Mouse ESCs grow on gelatin-coated support in the absence of feeder cells. To gelatin-coat a flask or plate, pour 1–2 mL of 0.2 % gelatin solution on the bottom of the flask, distribute homogeneously (the entire surface must be covered), incubate minimum 5 min at room temperature, and finally remove the gelatin (*see* **Note 4**).
4. Cell culture incubator: mESCs grow at 37 °C in 8 % CO_2.
5. Cell culture media: 0.05 % Trypsin containing EDTA (Life Technologies) and PBS pH 7.4 (1×) without $CaCl_2$ and $MgCl_2$ (Life Technology).
6. pX458 empty: Unmodified pSpCas9 (BB)-2A-GFP (pX458) vector at 500 ng/μL.
7. Specific CRISPR/Cas9 constructs: pSpCas9(BB)-2A-GFP vectors containing specific sgRNA (Obtained from Section 3.2) at 500 ng/μL.
8. Lipofection reagents: Opti-MEM® I reduced Serum (1×) (Life Technologies) (*see* **Note 5**) and Lipofectamine™ 2000 reagent (Life Technologies).
9. GenElute™ Blood Genomic DNA kit, Miniprep (Sigma).
10. PCR primers at a concentration of 10 μM.
11. Compounds for PCR reactions: 5× Green GoTaq® reaction buffer (Promega), dNTP mix 10 mM each (Thermo Scientific), GoTaq® G2 Polymerase (Promega), and autoclaved water.
12. Agarose powder (Invitrogen).
13. Ethidium Bromide Solution 10 mg/mL (Bio-Rad).
14. 2-Log DNA Ladder (0.1–10.0 kb) (New England BioLabs).
15. Electrophoresis DNA power supply and horizontal electrophoresis cell.
16. UV revelation device for DNA electrophoresis gel.

2.4 Lipofection of Cells with the Selected Combinations of Two Specific CRISPR/Cas9 Constructs and Isolation of Single Cells in 96-Well Plates

Items 1–8 are identical to Section 2.3.

9. Fluorescence-activated cell sorting (FACS) device/Moflo (Beckman Coulter) (*see* **Note 6**).
10. Dimethyl Sulfoxide (Sigma).

2.5 Genomic DNA Extraction from 96-Well Plates, PCR Screening and Identification of Potential Candidates

1. 96-well plates containing mESCs from Section 2.4.
2. Autoclaved water.
3. Lysis buffer: 10 mM Tris–HCl pH 7.5, 10 mM EDTA, 0.5 % SDS, 10 mM NaCl. Prepare 10 mL of 1 M Tris–HCl pH 7.5: weight 1.57 g of Tris–HCl (Applichem), dissolve it in 10 mL of autoclaved water and adjust the pH to 7.5. To prepare 50 mL of lysis buffer mix: 500 μL of 1 M Tris–HCl pH 7.5, 0.146 g of EDTA, 0.25 g of SDS, and 0.029 g of NaCl, complete to 50 mL with autoclaved water and mix. This buffer can be stored at room temperature.
4. Proteinase K (Applichem). Prepare a stock solution at 20 mg/mL: dilute 20 mg of proteinase K in 1 mL of autoclaved water. This stock solution must be stored at −20 °C.
5. Precipitation solution: 1.5 M NaCl. To prepare 15 mL of precipitation solution dissolve 1.31 g of NaCl in 15 mL of autoclaved water. This buffer can be stored at room temperature.
6. Ice cold EtOH 100 %.
7. EtOH 70 %.
8. 96-well PCR plates (Thermo Scientific).
9. PCR primers at a concentration of 10 μM.
10. Compounds for PCR reactions: 5× Green GoTaq® reaction buffer (Promega), dNTP mix 10 mM each (Thermo Scientific), GoTaq® G2 Polymerase (Promega).
11. Agarose powder (Invitrogen).
12. Ethidium Bromide Solution 10 mg/mL (Bio-Rad).
13. 2-Log DNA Ladder (0.1–10.0 kb) (New England BioLabs).
14. Electrophoresis DNA power supply and horizontal electrophoresis cell.
15. UV revelation device for DNA electrophoresis gel.

3 Methods

Many of the following procedures were adapted from a publicly available protocol by E.P. Nora and E. Heard (27).

3.1 Design of Paired CRISPR/Cas9 sgRNAs and PCR Primers

In this section, it is described how to design specific sgRNAs using two different online tools: the e-CRISP software (21) and the Feng Zhang lab's Target Finder (22). As previously mentioned in Section 1, a lot of CRISPR sgRNA design tools are now available, and any tools can be used as long as they take into account the prediction of off-targets (30).

Each sgRNA design software gives potential CRISPR target sites using a specific algorithm; in order to increase the chance to hit

genomic DNA, it is advised to use two distinct tools and to design two sgRNAs with each one (*see* **Note 2**).

In this protocol, the plasmid pSpCas9 (BB)-2A-GFP (pX458) (29) is used as vector and requires to add specific overhangs to the designed sgRNAs. Other vectors are available, and if another vector than pX458 is planned to be used, make sure to add the correct overhangs to insert the sgRNAs into the corresponding vector.

1. Delimit your genomic sequence of interest (*see* **Note 7**). It is advised to paste the DNA sequence of interest into a text editor of choice (e.g., Word) for further easy localization and handling of sgRNAs and PCR primer sequences.
2. Design sgRNAs using the e-CRISP online bioinformatics tool (21). Go to www.e-crisp.org and choose "De-novo". Then select "Mus musculus (Mouse, GRCm38.75)" as model organism using the drop-down list. Next, select "Enter Target Sequence" and enter the sequence of interest (FASTA format) in the corresponding box. Finally click on "Start sgRNA search". The website will provide a ranked list of potential sgRNAs, with a specificity score, an annotation score and their positions on the sequence of interest.
3. Choose two sgRNAs, which are within a 1–2 kb size range region (*see* **Note 8**) and locate them on your sequence of interest created at **step 1**.
4. Design sgRNAs using the Feng Zhang lab's Target Finder (22). Go to http://crispr.mit.edu/ and fill the required field: search name and e-mail address. Then select "mouse" as target genome and paste your sequence of interest into the corresponding box. Notice that a maximum of 250 bp can be pasted inside this box. It is advised to perform several rounds of research of 250 bp inside the 1–2 kb region delimited at **step 3**. Finally click on "Submit". The complete analysis takes 15 min in average, when the job is completed click on "Guides & off targets". The website will provide a ranked list of potential sgRNAs with a quality score, the number of off-target sites with the corresponding number and position of mismatches.
5. Choose two sgRNAs, which are within a 1–2 kb size range region delimited at **step 3** (*see* **Note 8**), and locate them on your sequence of interest created at **step 1**.
6. Design specific PCR primers using a PCR primer online software (*see* **Note 3**). For each sgRNA: design PCR primers in a region comprising at least 300 bp upstream and downstream of the sgRNA target site. Set up the parameters to amplify amplicons around 500–600 bp, at a melting temperature of 60 °C (*see* **Note 9**). Identify their positions on the sequence of interest.
7. Determine the expected PCR amplicon sizes for the deletion PCR. For each sgRNA couple combination (four distinct sgRNAs means six different combinations), determine the

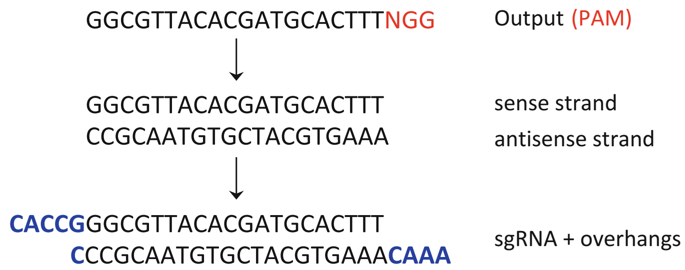

Fig. 2 sgRNA modifications for insertion into the pSpCas9(BB)-2A-GFP vector. sgRNAs have to be modified to assure a proper ligation with the digested vector backbone. The PAM sequence (*red*) needs to be removed in the first step and the sense strand complemented with its corresponding antisense. The ends need to be modified according to the restriction enzyme used for the vector. For BpiI (BbsI), this corresponds to a "CACCG" 5′ overhang of the sense strand and "CAAA" 5′/"C" 3′ overhang of the antisense strand (*blue*)

expected size for the deletion PCR product (*see* Fig. 1) knowing that the CRISPR/Cas9 complex cleaves the genomic DNA 3 nucleotides before the PAM motif (16).

8. Preparation for the ordering of sgRNAs and PCR primers. For each sgRNA: remove the PAM motif from the sequence, this is the sense strand (*see* **Note 10**). Then write the corresponding complement sequence, this is the antisense strand. Add a "CACCG" overhang on the 5′ of the sense strand. Flank a "CAAA" overhang on the 5′ and a "C" overhang on the 3′ of the antisense strand (*see* Fig. 2) (*see* **Note 11**). Order the sgRNAs and the PCR primers (*see* **Note 12**).

3.2 Construction of Specific CRISPR/Cas9 Vectors Using the pSpCas9(BB)-2A-GFP Plasmid

In this protocol, the pSpCas9(BB)-2A-GFP plasmid is used, also known as pX458 (Addgene plasmid ID: 48138). This plasmid allows the expression of a Cas9 nuclease fused with GFP. The plasmid pX458 is used at a concentration of 500 ng/μL.

1. Digest 1 μg of pX458 plasmid with BpiI (BbsI). Prepare two reactions, one containing the restriction enzyme and one negative control (uncut pX458 plasmid), as follows:

Stock	For one digestion reaction (μL)	For one negative control (μL)
Buffer G	2	2
BpiI (BbsI)	1	0
pX458 plasmid (500 ng/μL)	2	2
H_2O	15	16
Total (μL)	20	20

And incubate 1 h at 37 °C.

2. Prepare a 1 % Agarose gel with an Ethidium Bromide final concentration of 0.1 μg/mL. Add 4 μl of 6× loading dye to each sample and load the totality of the digestion reaction on the gel. Run the gel at 100 V for 45 min. Use the negative control as reference to excise the linearized plasmid and purify it using the QIAquick® Gel Extraction Kit. The linearized pX458 plasmid can be stored at –20 °C.
3. Generate the double stranded sgRNAs. Mix 8 μL of sense and antisense strand from one sgRNA in one PCR tube (one PCR tube per sgRNA design). Put the PCR tubes into a thermo cycler and run the following program:

Step 1: 94 °C for 4 min
Step 2: 70 °C for 10 min
Step 3: 37 °C for 20 min

4. Ligate the double-stranded sgRNAs into the BbsI digested vector. Additionally to the samples, prepare one negative control reaction that does not contain double stranded sgRNA. For one ligation reaction, mix the following reagents:

Stock	For one ligation reaction (μL)	For one negative control (μL)
Double-stranded sgRNA (annealed oligos)	2	0
Linearized pX458 plasmid (1 μg)	1	1
10× T4 DNA ligase buffer	2	2
T4 DNA ligase	1	1
H_2O	4	6
Total (μL)	10	10

Incubate overnight at room temperature.

5. Amplify the ligation products. For one ligation reaction: thaw one aliquot One Shot® TOP10 Chemically Competent *E. coli* on ice, then add 5 μL of ligation product to the competent cells, mix gently and incubate 15–30 min on ice. Heat shock bacteria 30 s at 42 °C in a water-bath and immediately put them back on ice. Then add 1 mL of room temperature LB without antibiotics and incubate 1 h at 37 °C under agitation (200 rpm). Centrifuge bacteria at 400 × *g* for 5 min, remove and discard 900 μl of supernatant, resuspend the bacteria pellet in the remaining 100 μl and plate on ampicillin agar plates

(one plate per condition). Finally incubate the plates overnight at 37 °C (*see* **Note 13**).

6. Validation of the cloning procedure. The day after, for each plate (except for the negative control plate), pick three single independent colonies and inoculate each of them into one bacterial culture tube containing 3 mL of LB with ampicillin at a final concentration of 0.1 mg/mL. Incubate at 37 °C under agitation (200 rpm) overnight. The day after, use the 2 mL of bacterial culture to extract plasmids for each condition using the GeneJET Plasmid Miniprep Kit, and keep the rest of the bacterial culture tubes at 4 °C. Send purified plasmids for sequencing using the following primer: 5′-GACTATCATAT GCTTACCGT–3′. Analyze the sequencing results and determine which bacterial clones present a correct sgRNA sequence incorporated into the pX458 vector (*see* **Note 14**).
7. Amplify the correct specific CRISPR/Cas9 constructs. Take out the bacterial culture tubes corresponding to the bacterial clones expressing the correct CRISPR/Cas9 constructs (determined at **step 6**). For each sample, prepare 1 L Erlenmeyer containing 200 mL of LB with ampicillin at a final concentration of 0.1 mg/mL. Then, pipet the 1 mL bacterial culture left at **step 6** into the corresponding 1 L Erlenmeyer. Incubate the 1 L Erlenmeyer at 37 °C under agitation (200 rpm) overnight. The day after, extract plasmid using the Pure Link™ HiPure Plasmid Filter Maxiprep Kit. Measure the DNA concentration of the samples and dilute them in autoclaved water to reach a final concentration of 500 ng/μL. Store the diluted DNA at –20 °C (*see* **Note 15**).

3.3 Efficacy Assay of Paired Designed CRISPR/Cas9 Constructs

For this part of the protocol, lipofection reactions are performed in 6-well plates (1 well per condition). There are eight different conditions: six different combinations of two specific CRISPR/Cas9 constructs and two negative controls. One negative control corresponds to cells transfected with the empty pSpCas9(BB)-2A-GFP and is referred in the protocol as “pX458 empty”. The other negative control corresponds to cells transfected with PBS (1×) instead of DNA and is referred in the protocol as “Mock”.

Mouse ESCs have been plated the day before, as cells must be between 50 and 70 % of confluence for the lipofection reaction.

1. Change the medium of the cells. Remove the mESC proliferative medium, and wash the cells with 2 mL of PBS (1×). Then add 2 mL of mESC proliferative medium without antibiotics (the antibiotics can inhibit the lipofection reaction). For each condition, prepare two 1.5 mL Eppendorf tubes labeled A and B.

2. For each A tube: put 240 μL of Opti-MEM® (1×) and then add 10 μL of Lipofectamine™ 2000 reagent. Mix gently by pipetting up and down and incubate 5 min at room temperature under the hood without light (*see* **Note 5**). During this time, prepare the B tube of each condition as follows:

Stock	For one reaction (μL)	Empty pX458 (μL)	Mock (μL)
Opti-MEM® (1×)	242	246	246
CRISPR/Cas9 construct 1 (500 ng/μL)	4	0	0
CRISPR/Cas9 construct 2 (500 ng/μL)	4	0	0
pX458 empty (500 ng/μL)	0	4	0
PBS (1×)	0	0	4
Total (μL)	250	250	250

3. For each condition: mix A and B gently by pipetting up and down. Then incubate 20 min at room temperature under the hood without light (*see* **Note 5**). Finally put the lipofection mix (500 μL) on each well and mix gently by pipetting up and down. Put the cells back in the incubator.
4. After 6–8 h, remove the medium from the cells, wash them with 2 mL of PBS (1×) and add 2 mL of new mESC proliferative medium for all conditions.
5. Change the medium of the cells 24 h after lipofection.
6. Harvest the cells 48 h after lipofection. For each well: aspirate the medium and wash once with 2 mL PBS (1×) then add 0.5 mL of 0.05 % Trypsin containing EDTA and incubate 5 min at 37 °C. Add 2 mL of mESC differentiation medium, pipet up and down and flush the well several times to detach all the cells. Then collect the cells in a 15 mL falcon tube and spin the cells 5 min at 180 × *g*. Aspirate the supernatant and resuspend the cell pellet in 1 mL PBS (1×). Then, transfer to a 1.5 mL Eppendorf tube. Finally spin the cells again 5 min at 180 × *g*, aspirate the supernatant and keep the cell pellet. Cell pellets must be kept on ice. If the following step in not performed the same day, store the cell pellets at −80 °C.
7. Extract DNA from cell pellets following the manual of the GenElute™ Blood Genomic DNA kit, Miniprep (Sigma). Measure DNA concentrations and dilute samples with autoclaved water to reach a DNA concentration of 50 ng/μL for all the samples (*see* **Note 16**).

8. Prepare the PCR tubes. For each of the CRISPR/Cas9 DNA samples, label three PCR tubes: 1F + 1R, 2F + 2R, and 1F + 2R. Then prepare for each tube: a corresponding "empty pX458" control containing "empty pX458" DNA, a corresponding "Mock" control containing "Mock" DNA, and a negative control PCR reaction containing water instead of DNA. This represents a total of 12 PCR tubes per combination of two specific CRISPR/Cas9 constructs (*see* Fig. 3).
9. Prepare PCR reactions as follows:

Stock	For one PCR reaction (μL)	For one negative control PCR reaction (μL)
5× Green GoTaq® reaction buffer	4	4
dNTP mix 10 mM each	0.4	0.4
Primer-F 10 μM	0.5	0.5
Primer-R 10 μM	0.5	0.5
GoTaq® G2 Polymerase	0.1	0.1
Sample's DNA (50 ng/μL)	1	0
H_2O	13.5	14.5
Total (μL)	20	20

10. Run the following PCR program:

Step 1: 94 °C for 5 min
Step 2: 94 °C for 30 s
Step 3: X°C for 30 s
Step 4: 72 °C for Y seconds
Step 5: GOTO step 2, 34 times
Step 6: 72 °C for 5 min
Step 7: 12 °C HOLD

X corresponds to the melting temperature of the primers, which have been designed previously. Y is specific for each primer combination and corresponds to the elongation time depending on the amplicon size that has been determined previously (Section 3.1, **step** 7) (*see* **Note 17**).

11. Prepare a 2 % Agarose gel with an Ethidium Bromide final concentration of 0.1 μg/mL. For each condition forecast 13 slots (*see* Fig. 3). Then load the gel with 10 μL of the PCR reactions. Store the rest at 4 °C (*see* **Note 18**). Reserve one slot

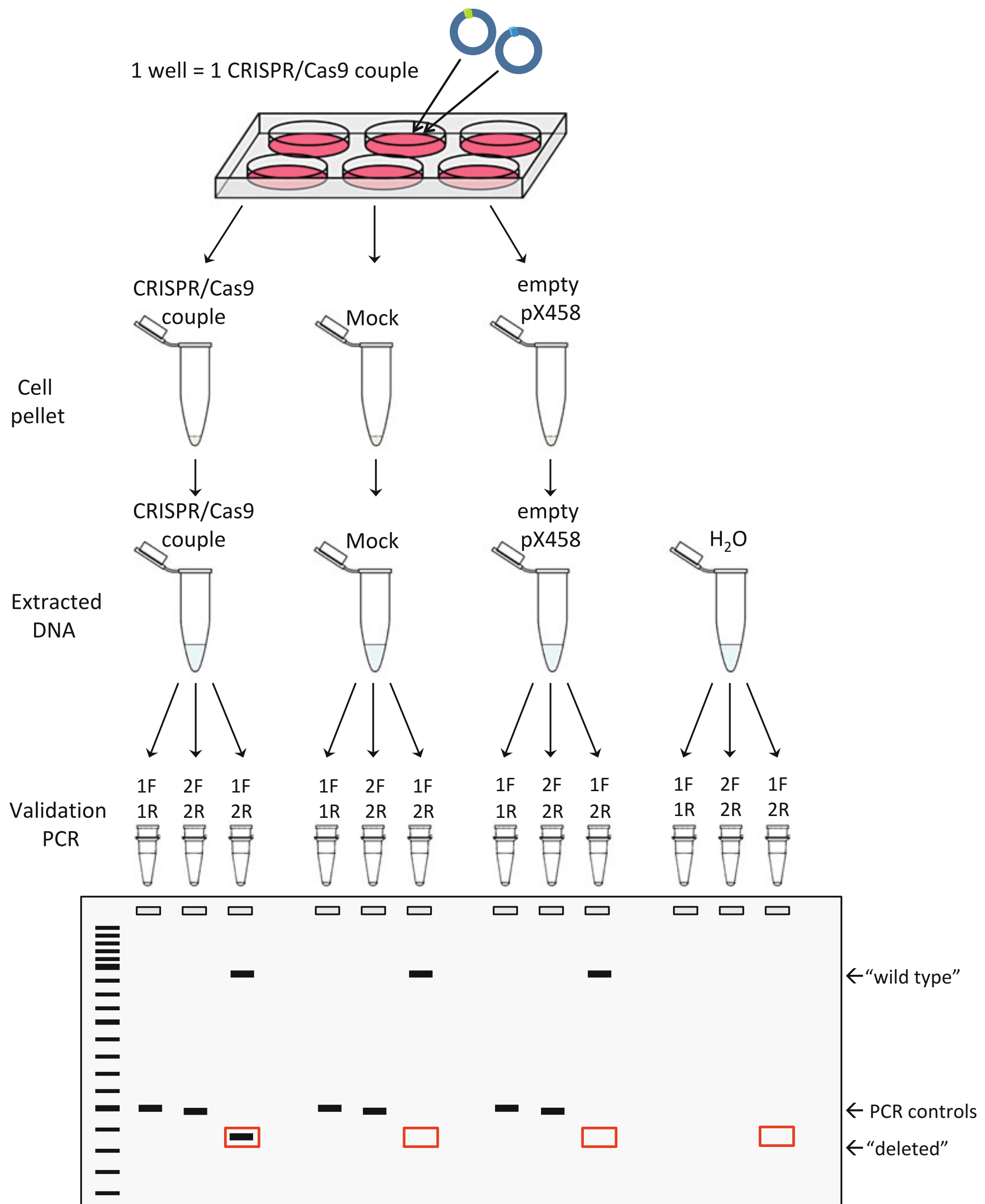

Fig. 3 PCR screen for CRISPR/Cas9 couple performance assessment. The figure shows consecutive steps to analyse one CRISPR/Cas9 couple in mixed population including empty pX458 vector-, mock-, and water-control (all steps, including controls have to be repeated for each CRISPR/Cas9 couple tested with the corresponding primers). 1F and 1R refer to specific primers flanking the sgRNA target site 1; 2F and 2R refer to specific primers of sgRNA target site 2. 1F + 1R as well as 2F + 2R are used as PCR primer control. Combination of the primers 1F and 2R allows the detection of the deletion induced by the paired CRISPR/Cas9 system. For the sample, two different scenarios are possible: (i) only one large amplicon corresponding to the "wild type" amplicon can be observed meaning that the deletion event did not occur, or (ii) one large and one small amplicon corresponding to the "wild type" and the "deleted" amplicon, respectively, can be observed, meaning that the deletion event occurred. The mock and empty pX458 controls must only display the "wild type" amplicon. No bands should be detected in the water control

to load 2.5 μL of 2-Log DNA Ladder. Run the electrophoresis (eg. 110 V for 1 h). Then reveal the Agarose gel using a UV device for DNA electrophoresis gel.

12. Analyze the results. Select the couples for which a band at the expected size appears for the deletion PCR but not for the "empty pX458" control, not for the "Mock" control, and not for the negative control PCRs (*see* Fig. 3). Only these couples will be used for the continuation of the protocol.

3.4 Lipofection of Cells with the Selected Combinations of Two Specific CRISPR/Cas9 Constructs and Isolation of Single Cells in 96-Well Plates

For this part of the protocol, lipofection reactions are performed in 6-well plates (1 well per condition). There are four different conditions: the two best combinations of two specific CRISPR/Cas9 constructs (determined in the previous step) and two negative controls: "empty pX458" and "Mock" (previously described in Section 3.3).

Here GFP expression is used as criteria to select the cells expressing the specific sgRNA pSpCas9(BB)-2A-GFP constructs. GFP expressing cells are FACS sorted and single cell distributed into 96-well plates (1 cell per well) in order to obtain single independent clones. Selecting only cells expressing GFP should increase the number of positive candidates with the specific knockout. The two negative controls are used to calibrate the cell sorter as the "Mock" control does not contain cells expressing GFP and the "empty pX458" does.

If no cell sorting device is available, an alternative method is proposed in Section 4 (*see* **Note 6**).

In this part of the protocol, 96-well plates are used, which implies a lot of pipetting steps that can be facilitated by the use of a multichannel pipette.

Mouse ESCs have been plated on 6-well plates the day before, as cells must be between 50 and 70 % of confluence for the lipofection reaction.

1. Transfect mESCs with the best combinations of two specific CRISPR/Cas9 constructs. Perform four lipofection reactions: one for each of the two best combinations of two specific CRISPR/Cas9 constructs (determined in the previous Section 3.3) and two negative controls: "empty pX458" and "Mock". For the lipofection procedure, follow exactly the **steps 1–5** described in Section 3.3.
2. Collect and sort the cells 48 h after lipofection. First, prepare eight gelatin-coated 96-well plates (50 μL of 0.2 % gelatin solution per well for minimum 5 min), remove the gelatin and add 200 μL of mESC proliferative medium per well. Then, for each well of the 6-well plate: aspirate the medium, wash the cells with 2 mL PBS (1×) then add 0.5 mL of 0.05 % Trypsin containing EDTA, and incubate 5 min at 37 °C. Add

2 mL of mESC differentiation medium, pipet up and down and flush the well several times to detach all the cells. Then collect the cells in a 15 mL falcon tube and spin the cells 5 min at 180 × *g*. Aspirate the supernatant and well resuspend the cell pellet in 1 mL of PBS (1×) + 2 % FBS. Keep the cells on ice until the sorting.

3. Sort the cells. Use the "empty pX458" cells and the "Mock" cells to calibrate the machine and sort 1 cell per well of the 96-well plates. For each "combination of two specific CRISPR/Cas9 constructs" cell sample, fill four 96-well plates. Then put the 96-well plates back in the incubator. If no cell sorting device is available, an alternative method is proposed in Section 4 (*see* **Note 6**).
4. Wait until the cells grow. If the medium's color changes, remove the medium and put 200 μL of fresh mESC proliferative medium per well. On average, it takes 15 days to observe a color change of the medium.
5. Regroup the clones from one "combination of 2 specific CRISPR/Cas9 constructs" on the same 96-well plate. For each combination: examine the plate under a microscope and identify full wells. Then pick all these clones into a new gelatin-coated 96-well plate containing 100 μL of mESC proliferative medium per well. For each clone: remove the medium, wash with 100 μL of PBS (1×), add 50 μL of 0.05 % Trypsin containing EDTA, incubate 5 min at 37 °C, then add 200 μL of mESC proliferative medium per well, pipet up and down and transfer everything into a well of the new 96-well plate.
6. Change medium 24 h after.
7. Wait until more than 50 % of the plate is around 70 % of confluence. Change medium if necessary. Then split each 96-well plate into two 96-well plates: one labeled as "freezing plate," the other one as "DNA plate". For one plate: prepare two new gelatin-coated 96-well plates containing 100 μL of mESC proliferative medium per well. Then for each well: remove the medium, wash with 100 μL of PBS (1×), add 50 μL of 0.05 % Trypsin containing EDTA. Then incubate 5 min at 37 °C, then add 100 μL of mESC proliferative medium per well, pipet up and down and transfer: 50 μL into a well of the "freezing plate" and 50 μL into a well of the "DNA plate".
8. Change medium 24 h after.
9. Wait until the cells grow. If the medium's color changes, remove it and put 200 μL of fresh mESC proliferative medium per well. When more than 50 % of the plate is around 50 % of confluence, the "freezing plate" can be frozen and the "DNA plate" is ready for DNA extraction.

10. Freeze cells in 96-well plates. For one well of a "freezing plate": remove the medium, wash with 100 μL of PBS (1×), add 25 μL of 0.05 % Trypsin containing EDTA, incubate 5 min at 37 °C, add 25 μL of FBS, and then add 50 μL of FBS + 20 % DMSO, pipet up and down. Finally seal the 96-well plate using parafilm, wrap the 96-well plate with tissues and store it at −80 °C.
11. Prepare 96-well plates for DNA extraction. For one well of a "DNA plate": remove the medium, wash with 100 μL of PBS (1×), then the DNA extraction can be performed or the plate can be sealed with parafilm and stored at −80 °C.

3.5 Genomic DNA Extraction from 96-Well Plates, PCR Screening and Identification of Potential Candidates

In this part of the protocol, DNA is extracted from the cells directly from 96-well plates. This implies a lot of pipetting steps, which can be facilitated by the use of a multichannel pipette.

1. If the cell plates have been stored at −80 °C, let them slowly thaw on ice. Otherwise directly proceed to the **step 2** with the samples.
2. Prepare 10 mL of complete lysis buffer. Add Proteinase K to lysis buffer at 1 mg/mL final concentration by mixing 500 μL of Proteinase K stock solution at 20 mg/mL with 9.5 mL of lysis buffer.
3. Lyse the cells. Add 50 μL of complete lysis buffer to each well, wrap plates with tissues and incubate at 60 °C, 4 h to overnight.
4. Extract DNA. Cool down plates on ice during 5 min, add 10 μL precipitation solution to each well and mix by pipetting. Then add 150 μL ice cold 100 % EtOH to each well, mix by pipetting and spin the plate 30 min at 4 °C at 2,500 × *g*. Invert plate to decant liquid and blot excess liquid on paper towels. Add 150 μL of 70 % EtOH to each well, mix by pipetting and spin the plate 15 min at room temperature at 2,500 × *g*. Again, invert plate to decant liquid and blot excess liquid on paper towels. Then, leave the plate open to dry completely at room temperature.
5. Resuspend DNA. Add 200 μL of autoclaved water to each well and incubate plates at least 1 h at 37 °C to resuspend DNA. These plates, designated as "undiluted DNA" plates, can be stored at −20 °C.
6. Dilute DNA. Prepare a 1/10 DNA dilution plate for each plate by distributing 45 μL of autoclaved water in new 96-well plates and adding 5 μL of "undiluted DNA" (*see* **Note 19**). These plates, designated as "diluted DNA" plates, can be stored at −20 °C.
7. Perform deletion PCR. For each plate, add 2 μL of "diluted DNA" to a 96-well PCR plate and prepare two master mixes,

one for each specific combination of primers for deletion PCR (corresponding to the CRISPR/Cas9 construct combination), as follows:

Stock	For one PCR reaction (μL)	Mix for 100 PCR reactions (μL)
5× Green GoTaq® reaction buffer	4	400
dNTP mix 10 mM each	0.4	40
Primer-F 10 μM	0.5	50
Primer-R 10 μM	0.5	50
GoTaq® G2 Polymerase	0.1	10
Sample's DNA at 50 ng/μL	1	–
H_2O	13.5	1350
Total (μL)	20	–

Add 19 μL of the master mix to the corresponding 96-well PCR plate containing DNA and mix by pipetting (*see* **Note 20**). Do not forget to perform a negative control for each master mix by mixing 19 μL of the master mix and 1 μL of autoclaved water.

8. Put the 96-well PCR plate tubes into a thermo cycler and run the following PCR program:

Step 1: 94 °C for 5 min
Step 2: 94 °C for 30 s
Step 3: X °C for 30 s
Step 4: 72 °C for Y seconds
Step 5: GOTO step 2, 34 times
Step 6: 72 °C for 5 min
Step 7: 12 °C HOLD

X corresponds to the melting temperature of the primers, which have been determined previously. Y is specific for each primer combination and corresponds to the elongation time depending on the amplicon size that has been determined previously (Section 3.1, **step** 7) (*see* **Note 17**).

9. Prepare a 2 % Agarose gel with an Ethidium Bromide final concentration of 0.1 μg/mL. Then load the gel with 10 μL of the PCR reactions. Store the rest at 4 °C (*see* **Note 18**). Reserve one slot to load 2.5 μL of 2-Log DNA Ladder and run the electrophoresis at 110 V for 1 h.

10. Analysis of the results. Identify the clones that present only the deletion band.
11. Thaw the candidate clones. As the freezing medium contains DMSO, which is toxic for the cells, once the "freezing plate" is taken out of the −80 °C, all the following steps have to be performed as fast as possible. For each candidate clone, transfer the cells from one well of the 96-well plate to one well of a 12-well plate.

    Gelatin-coat the number of 12-well plates you need according to the number of candidates clones you identified at **step 20**, then put 1 mL of mESC proliferative medium per well. It is advised to thaw at least 12 candidate clones, if possible. Take the "freezing plate" out of the −80 °C and add 200 μL of mESC proliferative medium to each candidate clone well, wait around 3 min until the medium is completely thawed. Then for each candidate clone, pipette up and down and transfer the entire content of one well of the 96-well plate to one well of a 12-well plate. When one 12-well plate is completed, put it in the cell incubator. From this point, candidate clones can be amplified and further tests for knockout confirmation can be performed at mRNA and protein level. The rest of the 96-well plate can be discarded.

4 Notes

1. Genomic DNA sequences are provided by the UCSC Genome Bioinformatics website (http://genome.ucsc.edu/).
2. Numerous sgRNA design tools are now available (29). However, another way to select sgRNAs is to choose already designed sgRNAs. For instance, Koike-Yusa's laboratory established a sgRNA library of 87′897 sgRNAs targeting 19′150 mouse protein-coding genes. The sgRNA sequences are provided in the supplementary data of the corresponding article (26).
3. To design specific PCR primers, the Primer-BLAST online software was used (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (31).
4. Gelatin-coated flasks or plates can be prepared in advance: pour few mL of 0.2 % gelatin on the bottom of the flask, distribute the gelatin homogeneously and keep them in the incubator (not more than 2 days to avoid evaporation). Five minutes before usage, take the plate out of the incubator and redistribute the gelatin homogeneously and do not forget to remove the gelatin solution before to add the cells.

5. Opti-MEM® I reduced Serum (1×) (Life Technologies) is a light sensitive product and must be kept from direct light exposure. It is advised to turn off the light of the cell culture hood during its use and wrap the bottle in aluminum foil for protection.
6. If FACS device is not available, an alternative method consists of diluting the cells and picking clones by "hand". Forty-eight hours after lipofection of the selected combination of two specific CRISPR/Cas9 constructs (Section 3.4, **step 2**), for each well of the 6-well plate: aspirate the medium, wash the cells with 2 mL PBS (1×) then add 0.5 mL of 0.05 % Trypsin containing EDTA, and incubate 5 min at 37 °C. Add 2 mL of mESC differentiation medium, pipet up and down and flush the well several times to detach all the cells. Then sample some of this cells suspension and assess its cellular concentration (number of cells per mL). In a new 15 mL Falcon tube, collect the right volume of cell suspension to have 2,000 cells and spin the cells 5 min at 180 × *g*. Aspirate the supernatant, well resuspend the cell pellet in 10 mL of mESC proliferative medium and transfer everything to a 10 cm gelatin-coated cell culture dish. Wait till cells proliferate. It should take around 1 week until clones can be seen by eye. During this waiting time, change the cell medium on a daily basis. Then for each condition process the following steps:
   (a) Prepare one gelatin-coated 96-well plate with 50 μL of 0.2 % gelatin solution per well (do not remove the gelatin) and one 96-well plate with 20 μL of 0.05 % Trypsin containing EDTA per well.
   (b) Take out the 10 cm dish containing the cells from the incubator and transfer the medium into a 15 mL Falcon tube. Then gently wash the 10 cm dish once with 5 mL of PBS 1(×).
   (c) Using a 10 μL pipette, take 10 μL of 0.05 % Trypsin from the first well of the first column on the 96-well plate containing the Trypsin. Select a colony on the 10 cm dish, place the pipette tip on the colony, pipet up and down until the colony comes off (around five times) and release the content of the pipette tip into the first well of the first column on the 96-well plate containing the Trypsin.
   (d) Repeat step (c) for all 8 wells of the first column on the 96-well plate containing the Trypsin. Be careful to not let the 10 cm dish dry more than 5 min, otherwise the cells will dry out and die. Filling one column of the 96-well plate must not take more than 5 min.

(e) Once one column is filled, transfer the medium from the 15 mL Falcon tube collected at step (b) to the 10 cm dish, and place the dish back in the incubator. Remove the gelatin from the first column of the gelatin-coated 96-well plate prepared at step (a). Then add 200 μL of mESC proliferative medium to each well of the first column of the 96-well plate containing the trypsinized cells, pipet up and down, transfer everything to the first column of the gelatin-coated 96-well plate and put this plate in the incubator. Be careful to not leave the cells more than 5 min in the 0.05 % Trypsin containing EDTA. Verify the presence of cells in the well with a microscope.

(f) Repeat steps (b–e) until the entire 96-well plate is filled. After step (e), it is advised to let the 10 cm dish containing the clones at least 5 min in the incubator to let the cells recover before a new round of picking. During these 5 min, the procedure can be performed on the other 10 cm dish (from the other specific CRISPR/Cas9 construct combinations). The two 10 cm dishes can be used alternatingly to avoid wasting time.

(g) Change the medium 48 h after (200 μL of mESC proliferative medium per well). Then change medium whenever the medium's color changes.

Then, go back to the protocol and pursue with Section 3.4, **step** 7.

7. It is advised to design your sgRNAs in order to target the gene in an exonic region ensuring the deletion is propagated to the mature RNA and does not accidentally get spliced out. It is also beneficial if the deletion is located close to the transcription start site as this avoids production of RNA fragments, which could have unknown side effects.
8. Choosing two sgRNAs, which are within a 1–2 kb size range region is not necessary but better for the PCR screening step, as this will allow to amplify the "wild type" PCR amplicon and the "deleted" PCR amplicon in the same PCR reaction. This will allow a quick detection and distinction between homozygote deleted mutants, heterozygote deleted mutants, and wild-type clones. It is possible to delete larger genomic regions. However, it is commonly observed that the larger the deletion is, the less efficient is the procedure (28).
9. PCR primers are designed in a way that they all have the same melting temperature. This makes them easier to combine for the PCR screening step.

10. Be careful, the sgRNA output sequence from the e-CRISP online bioinformatics tool includes the PAM motif. When modifying the sgRNAs by adding the specific BbsI overhangs and ordering the oligos, make sure that the PAM sequence has been removed.
11. The overhangs added to the sgRNAs make them compatible with the BpiI (BbsI) digested pX458 vector used in this protocol. In case it is planned to use another vector with different restriction site(s), do not forget to adapt the modifications of the sgRNAs according to the needs.
12. PCR primers and sgRNAs were ordered from Sigma. Basic desalting purification of primers and sgRNAs is sufficient enough for this protocol.
13. After overnight incubation at 37 °C, compare the number of colonies present on the negative control and sample plates. If there are no colonies on the sample plates, it could be due to a problem during the sgRNA annealing. Prepare a 5 % Agarose gel with an Ethidium Bromide final concentration of 0.1 μg/mL and load 5 μL of annealed sgRNAs (generated at Section 3.2, **step 3**) supplemented with 1 μL of 6× loading dye, next to 5 μl of single-stranded sgRNA (sense or antisense) supplemented with 1 μL of 6× loading dye. Then run the gel at 100 V for 1 h and verify the successful annealing by comparing the bands' sizes between annealed and nonannealed sgRNAs.
14. Serial Cloner (version 2-6-1) software was used to analyze sequencing results.
15. In order to save specific CRISPR/Cas9 constructs, it is possible to make glycerol stocks of the bacteria clones expressing the correct constructions. For each bacterial clone, take 750 μL of the 200 mL bacterial suspension (before to perform plasmid extractions using the Pure Link™ HiPure Plasmid Filter Maxiprep Kit) and mix it with 250 μL of 100 % autoclaved glycerol into a screw cap cryotube. Vortex and store the glycerol stocks at −80 °C.
16. If one (or several) sample(s) is (are) at a concentration lower than 50 ng/μL, equilibrate all the samples to the lowest sample concentration. Adjust the calculations to perform PCR reactions with 50 ng of DNA per reaction.
17. Concerning the elongation time of the deletion PCR (1F + 2R), choose an elongation time long enough to amplify the "wild type" amplicon. As this PCR reaction is performed on DNA extracted from a mixed population (transfected and nontransfected), if the CRISPR/Cas9 complex cleaved genomic DNA, two bands should be observed on the gel: one corresponding to the "wild type" amplicon and one lower in size, corresponding to the "deleted" amplicon.

18. Keeping 10 μL of the PCR reactions allow to have a backup in case of something goes wrong during the gel electrophoresis.
19. Be careful, the DNA solutions from the "undiluted DNA" plates are really viscous. Pipet slowly.
20. It is possible to run a positive control for this PCR by setting up a PCR reaction using DNA extracted from cells, which have been transfected with the corresponding combination of two specific CRISPR/Cas9 constructs (Section 3.3, **step** 7); or by loading the Agarose gel with the corresponding 10 μL of PCR reactions left at Section 3.3, **step 11**.

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CRISPR reagents are available to the academic community through Addgene (http://www.addgene.org/).

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Derivation of Neural Precursor Cells from Human Embryonic Stem Cells for DNA Methylomic Analysis

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Abstract

Embryonic stem cells are self-renewing pluripotent cells with competency to differentiate into all three-germ lineages. Many studies have demonstrated the importance of genetic and epigenetic molecular mechanisms in the maintenance of self-renewal and pluripotency. Stem cells are under unique molecular and cellular regulations different from somatic cells. Proper regulation should be ensured to maintain their unique self-renewal and undifferentiated characteristics. Understanding key mechanisms in stem cell biology will be important for the successful application of stem cells for regenerative therapeutic medicine. More importantly practical use of stem cells will require our knowledge on how to properly direct and differentiate stem cells into the necessary type of cells. Embryonic stem cells and adult stem cells have been used as study models to unveil molecular and cellular mechanisms in various signaling pathways. They are especially beneficial to developmental studies where in vivo molecular/cellular study models are not available. We have derived neural stem cells from human embryonic stem cells as a model to study the effect of teratogen in neural development. We have tested commercial neural differentiation system and successfully derived neural precursor cells exhibiting key molecular features of neural stem cells, which will be useful for experimental application.

Keywords: Human embryonic stem cells, Neural differentiation, Neural stem cell markers, DNA methylation

1 Introduction

Human embryonic stem cells (hESCs) are pluripotent self-renewable cells derived from the inner cell mass of mammalian blastocysts (1). They have the capacity to undergo indefinite rounds of cell division and differentiate into cells and tissues of all three germ layers (2). Differentiating hESC cultures provide a unique strategy to elucidate various molecular signals and processes during development. Differentiating hESC cultures into neural progeny may serve as a tool to elucidate mechanisms underlying early human neurogenesis. Such strategies may enable the development of in vitro models to allow for the systematic functional evaluation of developmental conditions and to decipher not only how such processes are regulated but also how they are altered by pathological conditions such as developmental disorders or exposure to neuroteratogens (3).

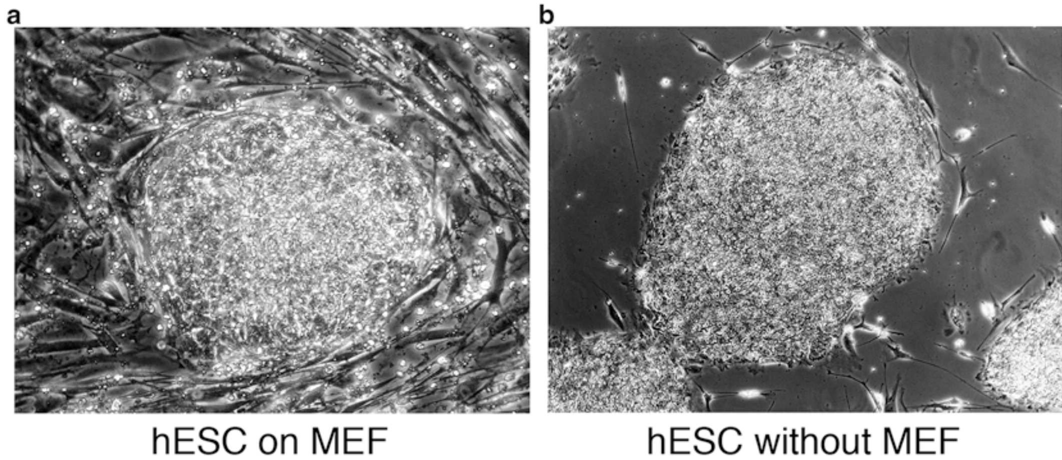

Fig. 1 Human embryonic stem cells (hESCs) cultured with MEF and without MEF. Early passage hESCs from frozen stock were cultured on mitotically inactivated mouse embryonic fibroblasts (MEF) in hESC growth medium supplemented with basic fibroblast growth factor (bFGF) as described in Sect. 3. When reached the confluency of 80–90 %, the cells were transferred to the feeder-free hESC culture medium and propagated. (**a**) hESCs grown on MEF feeder and (**b**) hESCs cultured on the feeder-free mTeSR1 medium. The first-passage cells are shown with some residual MEF

The hESC is typically grown on mouse embryonic fibroblast (MEF) feeder cells in hESC medium, and directed differentiation of hESC to neural stem cells is commonly performed in an adherent culture (4). Neural differentiation factors such as retinoic acid (RA), noggin, and B27 are also commonly added to the medium (5). After about a week of differentiation, feeder cells are removed and replaced with a neural proliferation medium. By this time, cells committed to their neural fates demonstrate neural rosettes in the culture (6). Cells are then cultured with a neural permissive medium and rosettes are passaged in medium containing high amounts of the mitogens FGF-2 and EGF (7). Neural progenitors (NP) can then be maintained for an unlimited time in the presence of FGF-2 and EGF. In culture, these newly formed NP maintain expression of SOX2 and begin expressing additional NP markers such as nestin, A2B5, SOX1, SOX3, and PSA-NCAM (8, 9).

Conventional hESC culture method involves the use of mouse embryonic fibroblast (MEF) feeder cells. The presence of contaminating MEF in the culture may present some issues in downstream application using hESCs. To avoid this potential pitfall, a feeder-free culture system for hESC has been developed by providing a supporting matrix and necessary factors for maintaining the potency of hESCs. For some of our applications, we need to have hESC samples devoid of any contaminating MEF. We initially maintained hESCs on the conventional MEF culture (Fig. 1a) and transferred the cells to the feeder-free mTeSR1 culture system (Fig. 1b). After a couple of passaging we found the culture was free of any MEF cells. We also found that hESC maintained its

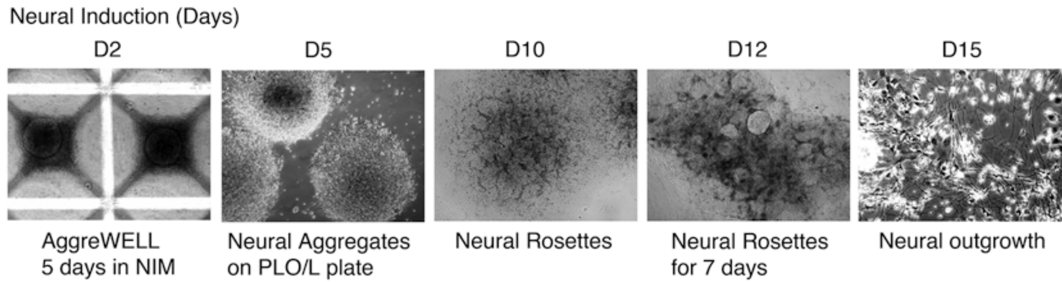

Fig. 2 Neural differentiation of hESC in vitro. Human embryonic stem cells were subjected to embryoid body (EB) formation using AggreWell for 5 days in neural induction medium (NIM). Neural aggregates were seeded on plates coated with poly-L-ornithine/laminin (PLO/L) and cultured with NIM for additional 7 days to develop neural rosette structure. After 7 days, the neural rosettes were dislodged and then replated for the expansion of neural precursor cells for 3–5 days

characteristic self-renewal and pluripotent properties as assessed by several molecular criteria (data not shown).

hESCs cultured on MEF feeder-free system were subjected for neural differentiation by using STEMdiff Neural System (STEMCELL Technologies, Inc., Vancouver, Canada). To achieve efficient and uniform embryoid body (EB) formation, AggreWell (STEMCELL Technologies, Inc., Vancouver, Canada) was used. As shown in Fig. 2, this method enabled us to obtain uniform size EBs. After 5 days of EB formation in neural induction medium, EBs were harvested and transferred to cell culture vessel coated with poly-L-ornithine and laminin. Cells were further cultured in neural induction medium for 7 days and neural rosettes were developed (Fig. 2). The neural rosettes were dislodged and replated for the expansion of neural precursor cells. After 3–5 days of expansion, cells were collected and preserved as the first-generation neural precursor cells (NPC0). NPC0 were further expanded by using neural expansion medium. To confirm proper differentiation into neural lineage cells, we have performed qRT-PCR analysis and immunofluorescence (IF) analysis on neural rosettes and NPCs (Fig. 3). Figure 3a shows induced expression of neural markers, nestin and tubulin, in rosettes and NPCs. IF analysis showed significant increase of nestin expression in neural rosettes and NPCs (Fig. 3b). SOX expression remained high in pluripotent hESCs and also neural cells.

Our study objective was to profile DNA methylomic changes in hESC-derived neural precursor model. To enrich methylated genomic DNA, we have performed methylated DNA immunoprecipitation (MeDIP) analysis as described in Sect. 2. Total genomic DNA was isolated and fragmented into pieces of mainly 500 bp (Fig. 4a). Fragmented DNA was immunoprecipitated with anti-5-methyl cytosine antibody (Eurogentec, Osaka, Japan) and purified. The resulting DNA was further amplified with whole genomic DNA amplification kit to obtain enough amount of DNA for next-generation DNA sequencing analysis.

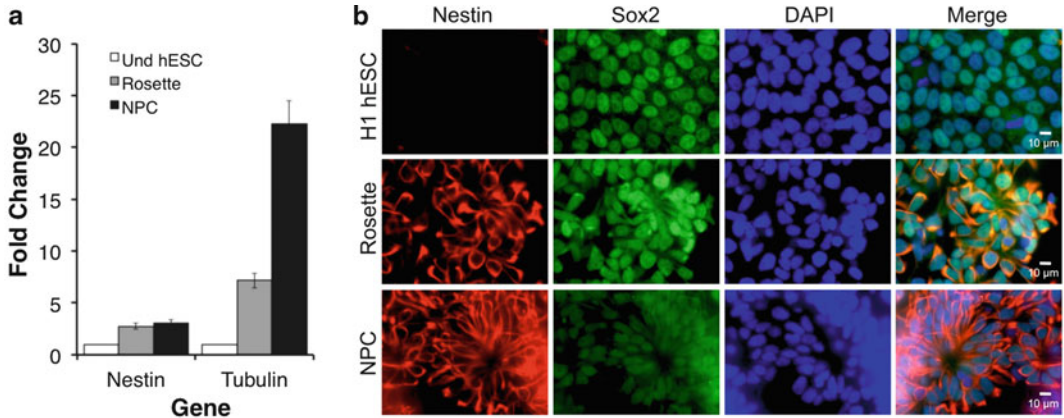

Fig. 3 Induced expression of neural markers in hESC-derived neural stem cells. Neural differentiation of hESCs was confirmed by examining the expression of neural markers. (**a**) Quantitative RT-PCR analysis was performed for *nestin* and *tubulin*, and the level of expression was compared among undifferentiated hESCs, rosettes, and NPCs. There was significant ($p < 0.05$) induction of both *nestin* and *tubulin* mRNA in neural cells derived from hESCs. (**b**) The induced expression of neural marker expression was also confirmed by immunofluorescence analysis. Nestin expression was noticeably increased in rosettes and NPCs. SOX2 expression remained high in pluripotent hESCs and also hESC-derived neural lineage cells. Cells were counterstained with DAPI for nuclei (scale bar, 10 μm with 60× magnification)

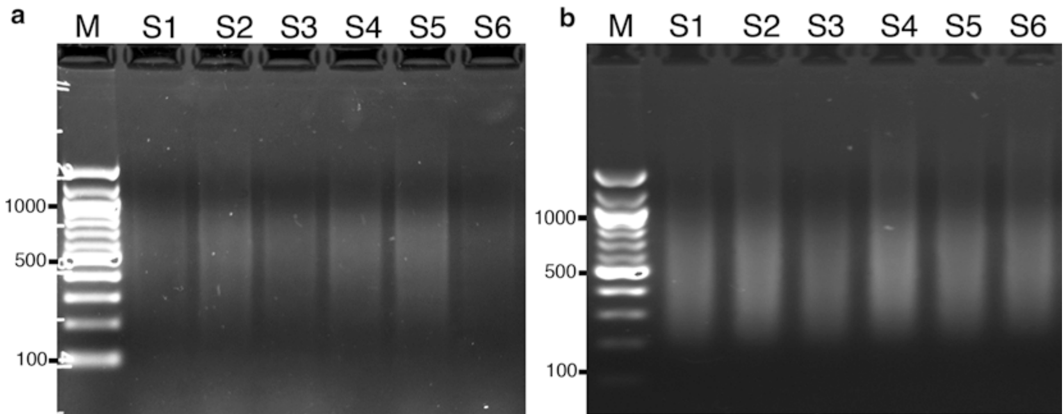

Fig. 4 Preparation of total genomic DNA and methylated DNA immunoprecipitation. (**a**) Total genomic DNA samples from hESCs were fragmented by sonification. (**b**) Fragmented genomic DNA was subjected to methylated DNA immunoprecipitation (MeDIP). The resulting immunoprecipitated DNA was amplified by using whole genomic DNA amplification kit

2 Materials

2.1 Plasticware and Chemicals

1. Tissue culture plates 100 mm and six-well plates.
2. 1, 5, 10, and 25 ml pipettes.
3. 0.22 μm syringe filter and disposable syringe.

4. 0.45 μm filter unit (Corning Inc., Corning, NY).
5. Cell scraper (Thermo Fisher Scientific, Pittsburgh, PA).
6. AggreWell (STEMCELL Technologies, Inc., Vancouver, Canada).

2.2 Culture and Propagation of hESCs

1. Dulbecco's Modified Eagle's Medium (DMEM)/F-12 (Invitrogen, Carlsbad, CA).
2. Knockout serum replacement (KSR), L-glutamine, nonessential amino acids, β-mercaptoethanol, 0.25 % trypsin-EDTA, 100× Pen Strep (all purchased from Invitrogen/Life Technologies, Carlsbad, CA).
3. Basic fibroblast growth factor (bFGF, R&D Systems, Minneapolis, MN): Dissolve 50 μg bFGF in 5 ml 0.1 % BSA/PBS to make a 10 μg/ml stock. Aliquot into 500 μl portions. Store at −80 °C.
4. Collagenase IV solution (1 mg/ml): Add the collagenase IV powder (50 mg) to a 50 ml falcon tube. In the hood, mix in 50 ml of sterile DMEM/F12 medium. Vortex the solution for <1 min. to make sure the powder is dissolved. In the hood, pass through a 0.22 μm filter into a new sterile 50 ml falcon tube. This solution is good for a maximum of 2 weeks stored at 4 °C.
5. mTeSR1 medium (STEMCELL Technologies, Inc., Vancouver, Canada).
6. ROCK inhibitor (Y-27632, Chemdea, Ridgewood, NJ).
7. Matrigel (BD Matrigel, hESC-Certified, LDEV-free, BD Biosciences, San Jose, CA).
8. 1× Accutase (STEMCELL Technologies, Inc., Vancouver, Canada).
9. Dispase (5 mg/ml, STEMCELL Technologies, Inc., Vancouver, Canada). Dilute dispase to 1 mg/ml using DMEM/F12. This solution can be kept for 1 week at 4 °C.
10. 1× phosphate-buffered saline (PBS, Invitrogen, Carlsbad, CA).

2.3 Preparation of hESC Culture and Neural Differentiation Medium

1. hESC growth medium (hESGM) for culturing with mouse embryonic fibroblast feeders was prepared using DMEM/F12 (200 ml) with 20 % KSR, 1 % nonessential amino acids, 1 mM L-glutamine, and 1 mM β-mercaptoethanol. The prepared hESGM was filter sterilized by using 0.45 μm filter unit and kept at 4 °C. Basic FGF was added to 5–10 ng/ml before using hESGM.
2. Feeder-free hESC culture medium was prepared by adding 100 ml of 5× supplement to mTeSR1.
3. STEMDiff neural induction medium (NIM) was used for neural induction of hESCs.

2.4 Isolation of RNA and Quantitative RT-PCR Analysis

1. RNeasy mini kit (Qiagen, Valencia, CA).
2. RNase-free TURBO DNase (Ambion, Austin, TX).
3. iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA).
4. Roche LightCycler 480 SYBR Green I Master mix (Roche, Indianapolis, IN).
5. PCR Primers.

   Nestin: Forward CGTTGGAACAGAGGTTGGAG.

   Reverse AGGCTGAGGGACATCTTGAG.

   Tubulin: Forward CGCCCAGTATGAGGGAGAT.

   Reverse GAGGCCTCGTTGTAGTAGACG.

   GAPDH: Forward CCTGCACCACCAACTGCTTA.

   Reverse GGGCCATCCACAGTCTTCTG.

2.5 Immunofluorescence Microscopy

1. Coverslips and glass slides.
2. 4 % paraformaldehyde.
3. 1× PBS.
4. Tris-buffered saline with Tween 20.
5. Primary antibodies.
6. Secondary antibody.
7. VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA).
8. Nail polish.
9. Olympus IX81 motorized inverted microscope with fluorescence filters.

2.6 Isolation of Genomic DNA

1. PureLink genomic DNA isolation kit (Invitrogen/Life Technologies, Carlsbad, CA).
2. Tris-EDTA, pH 8.0 (Ambion, Austin, TX).

2.7 Methylated DNA Immunoprecipitation (MeDIP)

1. Sonicator (Thermo Fisher Scientific, Pittsburgh, PA).
2. Anti-methyl cytosine (5mC) antibody (Eurogentec, Cat #BI-MECY-1000, Osaka, Japan).
3. Dynabeads (M-280 Sheep anti-mouse IgG, Life Technologies, Grand Island, NY).
4. Whole genome amplification kit (Sigma-Aldrich, St. Louis, MO).
5. Proteinase K (Invitrogen, Carlsbad, CA).
6. Glycogen (Thermo Fisher Scientific, Pittsburgh, PA).
7. Phenol:chloroform:isoamyl alcohol (25:24:1, USB Products, Cleveland, OH).

3 Methods

3.1 Culture and Propagation of Human Embryonic Stem Cells

3.1.1 Culturing hESCs

1. Early passage H1 and H9 human embryonic stem cells (passes 35–37) were obtained from UCLA Broad Stem Cell Research core facility through a license agreement with WiCELL Research Institute (Madison, WI). The cells were provided as growing on mouse embryonic fibroblast (MEF) feeder cells.
2. Cells were cultured at 37 °C with 5 % CO_2 in humidified incubator.
3. Cells were fed everyday with fresh complete hESGM supplemented with bFGF till confluent.

3.1.2 Propagation of hESCs in Feeder-Free Culture Medium

Cells cultured on mouse embryonic fibroblast feeder layer are transferred to mTeSR1 feeder-free human embryonic stem cell (hESC) culture system (STEMCELL Technologies, Inc., Vancouver, Canada).

1. Coat the cell culture vessel with diluted Matrigel (diluted 1:100 in DMEM/F12) for >1 h at RT (**Note 1**).
2. Confluent hESCs grown on six-well plates are washed once with prewarmed DMEM/F12.
3. Add 1 ml of collagenase on each well and incubate for 3–5 min at 37 °C.
4. Observe under the microscope for the sign of curling up of the cell boundary.
5. Wash the cells twice with prewarmed DMEM/F12.
6. Add 2 ml of prewarmed DMEM/F12 per well and use a cell scraper to gently scrape off the cells and collect in 15 ml tube. Add 2 ml of DMEM/F12 per well and collect all in 15 ml tube.
7. Spin cells for 3 min at 300 × *g* and remove the medium.
8. Resuspend the cells in 10 ml of prewarmed mTeSR1.
9. Cells are seeded and cultured with a daily medium change.

3.2 Derivation of Neutral Stem Cells from hESCs

Cultured cells were subjected to neural differentiation by using STEMdiff Neural System (STEMCELL Technologies, Inc., Vancouver, Canada) according to the manufacturer's instruction.

1. Exponentially growing cells are washed once with PBS and dissociated by treating with 1× Accutase (STEMCELL Technologies, Inc., Vancouver, Canada) for 5 min at 37 °C.
2. Cells are collected into 50 ml Falcon tube and spin for 5 min at 300 × *g*.
3. Cell pellets are washed twice with Dulbecco's Modified Eagle's Medium/F-12 (DMEM/F12) and finally resuspended in

neural induction medium (NIM) containing 10 μM Y-27632 (Chemdea, Ridgewood, NJ).

4. Cell suspension is subjected to embryoid body formation by using AggreWell 800 plate (STEMCELL Technologies, Inc., Vancouver, Canada).
5. Each well is rinsed with 1 ml of DMEM/F12 and aspirate to remove.
6. STEMdiff NIM supplemented with 10 μM Y-27632 (0.5 ml per well) is added to each well.
7. The plate is briefly centrifuged at 2,000 × *g* for 5 min to remove any air bubbles from the microwells and observed under a microscope to make sure that bubbles have been removed.
8. Cells in single suspension (2–3 × 10^6 cells) are added per well and the plate is centrifuged at 100 × *g* for 3 min to capture cells in the microwells.
9. The plate is examined under a microscope to confirm cells were evenly distributed among the microwells.
10. The next day, cells are fed with fresh NIM without Y-27632.
11. Neural aggregate formation is done for 5 days at 37 °C, 5 % CO_2 with a partial medium (3/4 of culture medium) change every day.
12. For culture of neural aggregates, six-well culture plates are coated with poly-L-ornithine (15 μg/ml in PBS, Sigma Catalog #P4957) for 2 h at room temperature and washed twice with PBS and once with DMEM/F12.
13. The plates are then coated with laminin (10 μg/ml in ice-cold DMEM/F12, Sigma Catalog #L2020) overnight at 4 °C.
14. The laminin solution is aspirated and the neural aggregates harvested are transferred into the well coated with PLO/L.
15. The cells are cultured at 37 °C with 5 % CO_2 and 95 % humidity with a full medium change daily for 7 days with STEMdiff NIM.
16. Morphological assessment and scoring of neural rosettes is done to ensure 50 % or more of the area of each aggregate is filled with neural rosettes.
17. On day 7 of attached neural aggregate culture, neural rosettes are selected away from contaminating flat cells.
18. The medium is removed from each well and washed with 1 ml of DMEM/F12 per well. STEMdiff Neural Rosette Selection Reagent (1 ml) was added per well and incubated for 1 h at 37 °C.
19. The STEMdiff Neural Rosette Selection Reagent is removed by using a micropipette outfitted with a disposable 1 ml tip.

20. The attached aggregates ware detached from the plates by expelling prewarmed DMEM/F12 onto the rosette clusters using a micropipette outfitted with a disposable 1 ml tip.
21. Detached neural rosettes are collected and centrifuged for 5 min at 350 × *g*.
22. The rosettes are resuspended in prewarmed NIM and briefly pipetted up and down and plated onto six-well plates precoated with PLO/L.
23. Cells are cultured at 37 °C with 5 % CO_2 and 95 % humidity with daily full medium changes using prewarmed STEMdiff NIM for 5 days (**Note 2**).
24. To ensure proper neural differentiation of hESCs, the same experimental procedure is applied to a set of cells plated on the coverslips. The level of neural markers (nestin, SOX2, musashi, and βIII tubulin) is assessed by immunofluorescence microscopy and quantitative RT-PCR analysis.

3.3 Immunofluorescence Microscopy

1. Carefully aspirate the medium and fix the cells with a fixative (i.e., 4 % paraformaldehyde in 1× PBS). Be careful to not aspirate the cells.
2. Incubate in 4 % paraformaldehyde for 15–20 min at room temperature.
3. Carefully aspirate the fixative and rinse three times (5–10 min each) with 1× PBS.
4. Apply a blocking solution (5 % Normal donkey serum, 0.3 % Triton X-100 in 1× PBS) for at least 2 h at room temperature or overnight at 4 °C. *Do not shake the cells.*
5. Dilute the primary antibodies included in this kit to working concentrations in the appropriate blocking solutions (mouse anti-nestin, 2 μg/ml; rabbit anti-SOX 2, 1 μg/ml).
6. In a separate control well, depending upon the specific antibody used, add equivalent concentrations of mouse IgG (1 mg/ml) or rabbit IgG (1 mg/ml).
7. Incubate the cells in primary antibodies overnight at 4 °C. *Do not shake.*
8. The next day, wash the cells twice with 1× PBS (5–10 min each wash) and twice with the appropriate blocking solution.
9. At the completion of the last wash, leave the cells in blocking solution for at least 30 min.
10. Dilute secondary antibodies at a 1:250 to 1:500 dilution in the appropriate blocking solution just before use.
11. Overlay the cells with the appropriate donkey anti-mouse or anti-rabbit secondary antibody that is conjugated to fluorescent molecules for 2 h at room temperature.

12. Wash three to five times (5–10 min each) with 1× PBS.
13. Mount a glass coverslip over the slides using VECTASHIELD anti-fading mounting solution.
14. Visualize the cell staining with a fluorescent microscope.

3.4 Quantitative RT-PCR Analysis

1. Total RNA is isolated by using Qiagen RNeasy mini kit and concentration is determined by NanoDrop.
2. To remove any contaminating genomic DNA, 10 μg of total RNA is subjected to DNase treatment.
3. Add 0.1 volume of 10× TURBO DNase buffer and 1 μl of TURBO DNase to the RNA, and mix gently.
4. Incubate at 37 °C for 30 min.
5. Add 0.1 volume of resuspended DNase inactivating reagent and mix well.
6. Incubate 10 min at RT with mixing occasionally.
7. Centrifuge at 10,000 × *g* for 1.5 min and transfer the RNA to a fresh tube.
8. Measure RNA concentration by NanoDrop.
9. Equal amount of RNA is subjected to cDNA synthesis using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) by mixing 4 μl of 5× iScript reaction mix, 1 μg of total RNA in 15 μl, and 1 μl of iScript reverse transcriptase in total volume of 20 μl.
10. Incubate the reaction mix for 5 min at RT, 30 min at 42 °C, and 5 min at 85 °C.
11. The resulting cDNA is diluted to 1:10 using RNase/DNase-free H_2O.
12. Quantitative PCR is done using Roche LightCycler SYBR Green I Master Mix by mixing 1.5 μl of cDNA, 5 μl of 2× Master mix, and 2 μl of diluted PCR primer pairs (10 μM each) in total 10 μl per reaction for 384-well platform.

3.5 Preparation of Total Genomic DNA

1. Total genomic DNA is isolated by using PureLink genomic DNA isolation kit (Invitrogen/Life Technologies, Carlsbad, CA).
2. Measure DNA concentration by using NanoDrop 1000 spectrophotometer (NanoDrop products, Wilmington, DE).
3. Genomic DNA is randomly sheared by sonication to generate fragments between 300 and 1,000 bp (**Note 3**).
4. Dilute the genomic DNA in TE in a 1.5 ml Eppendorf tube (10–20 μg DNA in 400 μl TE, 40–60 μg DNA in 700 μl TE).
5. Sonicate five times 10 s (BRANSON digital Sonifier model 450, used with the tapered Microtip, amplitude 20 %), with 1 min intervals between pulses (keep the tube on ice during the sonication).

6. Load 5 μl of sonicated DNA on an agarose gel to check the size of the DNA (mean size should be 300–1,000 bp).
7. If necessary, sonicate one or two additional pulses until the size of the DNA is 300–1,000 bp.
8. Precipitate the sonicated DNA with 400 mM NaCl, glycogen (1 μl), and 2 volumes 100 % ethanol.
9. Resuspend the DNA pellet in water and measure DNA concentration by NanoDrop.

3.6 Methyl DNA-Specific Immunoprecipitation (MeDIP)

The sonicated DNA is then immunoprecipitated with a monoclonal antibody against 5-methylcytidine (5mC) (Eurogentec Cat #BI-MECY-1000). A portion of the sonicated DNA should be left untreated to serve as input control.

1. Dilute 4 μg of sonicated DNA in 450 μl TE.
2. Denature for 10 min in boiling water and immediately cool on ice for 10 min.
3. Add 50 μl of 10× IP buffer (100 mM Na phosphate pH 7.0, 1.4 M NaCl, 0.5 % Triton X-100).
4. Add 10 μl of 5mC antibody.
5. Incubate >2 h at 4 °C with overhead shaking.
6. Prewash 40 μl of Dynabeads with 800 μl of PBS-BSA 0.1 % for 5 min at RT with shaking.
7. Collect the beads with a magnetic rack and repeat wash with 800 μl PBS-BSA 0.1 %.
8. Collect the beads with a magnetic rack and resuspend in 40 μl of 1× IP buffer.
9. Add Dynabeads to the sample and incubate 2 h at 4 °C with overhead shaking.
10. Collect the beads with a magnetic rack and wash with 700 μl 1× IP buffer for 10 min at RT with shaking.
11. Repeat wash with 700 μl 1× IP buffer twice.
12. Collect the beads with a magnetic rack and resuspend in 250 μl proteinase K digestion buffer.
13. Add 7 μl proteinase K (10 mg/ml stock).
14. Incubate 3 h at 50 °C (use a shaking heating block 100 × *g* to prevent sedimentation of the beads).
15. Extract with 1 volume phenol:chloroform:isoamyl alcohol (25:24:1) (500 μl).
16. Precipitate the DNA with 400 mM NaCl (20 μl of NaCl 5 M), glycogen (1 μl), and 2 volumes of 100 % ethanol (500 μl).
17. Resuspend the DNA pellet in 60 μl TE and keep at −20 °C.

3.7 Amplification of Methylated DNA After MeDIP

1. Methylated DNA is amplified by using whole genome amplification kit (Sigma-Aldrich, St. Louis, MO).
2. Add 2 μl of 1× Library Preparation Buffer to each sample.
3. Add 1 μl of Library Stabilization Solution.
4. Cool the sample on ice, consolidate by centrifugation, and return to ice.
5. Add 1 μl of Library Preparation Enzyme, vortex thoroughly, and centrifuge briefly.
6. Incubate the sample in a thermal cycler for 20 min at 16 °C, 20 min at 24 °C, 20 min at 37 °C, and 5 min at 75 °C, and hold at 4 °C.
7. Remove samples from thermal cycler and centrifuge briefly. Samples may be amplified immediately or stored at −20 °C for 3 days.
8. For amplification, a master mix may be prepared by adding 7.5 μl of Amplification Master Mix, 47.5 μl of Water Molecular Biology Reagent, and 5 μl of WGA DNA Polymerase to the 15 μl reaction from step 7.
9. Vortex thoroughly and centrifuge briefly.
10. The following profile has been optimized for a PE 9700 or equivalent thermocycler: initial denaturation at 95 °C for 3 min and 14 cycles as follows. Denature at 94 °C for 15 s and anneal/extend at 65 °C for 5 min.
11. After cycling is complete, maintain the reactions at 4 °C or store at −20 °C until ready for analysis or purification (**Note 4**).

4 Notes

1. Matrigel stock should be thawed and kept on ice for dilution. Diluted Matrigel should be kept on ice during experiment and can be stored at 4 °C for 2 weeks.
2. Derived NPC can be further expanded by using STEMdiff Neural Progenitor Medium (Cat# 05833, STEMCELL Technologies, Inc., Vancouver, Canada) or StemPro neural stem cell serum-free medium consisting of KnockOut DMEM/F-12 with StemPro Neural Supplement, EGF (20 ng/ml), bFGF (20 ng/ml), GlutaMAX-I (2 mM), and StemPro Neural Supplement (Life Technologies, Grand Island, NY).
3. The sonication efficiency varies with DNA concentration, sonicator settings, and size and quality of the sonication tip; therefore, it is recommended to systematically check the size of the sheared DNA to ensure equal sonication between experiments.

4. Amplified DNA should be purified by phenol extraction and quantified by using NanoDrop. DNA should be analyzed by agarose gel electrophoresis. If the amount of DNA is not sufficient, a second round of amplification can be performed by repeating PCR amplification with a small volume of amplified DNA.

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26S and PA28-20S Proteasome Activity in Cytosolic Extracts from Embryonic Stem Cells

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Abstract

The proteasome is a complex multisubunit protease that plays a major role in the degradation of proteins in eukaryotic cells. Proteasome function is one of the key players regulating the proteome and it is vital for many cellular processes. The method described here makes it possible to assay the proteolytic capacities of proteasome complexes separately in crude cytosolic extracts from ES cells. The method is based on hydrolysis of a fluorogenic peptide substrate in lysates prepared under conditions that favor the interactions of the 20S proteasomal catalytical core with either the 19S or the PA28αβ proteasome regulator.

Keywords: Proteasome, Proteasome activity, 19S proteasome activator, PA28 proteasome activator, Embryonic stem cells, Cell differentiation

1 Introduction

The proteasome is a complex multisubunit protease composed of the 20S catalytical core and a number of alternative proteasome regulators that physically interact with 20S and regulate substrate access to its proteolytic chamber. The 20S proteasome has a hollow cylindrical structure composed of one ring of α-subunits on each side of two inner β-subunit rings. Proteolysis is carried out by β-subunits while the α-subunits regulate substrate entry and binding of 20S regulators.

Degradation by the proteasome can be dependent on ATP and ubiquitination of the protein substrate. This type of protein degradation requires specific binding of the 20S proteasome to the regulator 19S forming the 26S proteasome (1). In contrast, 20S activity upon binding with the regulator PA28αβ is independent of both ATP and ubiquitin. The PA28-20S complex is associated to the production of antigen peptides (2) and protein homeostasis upon oxidative stress (3, 4). PA28αβ is predominantly cytosolic while 19S is found both in the cytosol and the nuclei (5). Other nuclear regulators of the 20S catalytical core are represented by PA28γ, which is important for cell cycle control (6), and PA200, involved in DNA repair (7). In addition, hybrid proteasome

variants where a 19S regulator binds 20S on one side while either PA28αβ or PA200 is occupying the other have also been described and add another layer of complexity in 20S regulation (8–10).

Proteasome activity can be assayed within cells using various substrates like GFPu/GFPdgn (11), ΔssCPY*-GFP (12), or the ubiquitination-independent GFP-odc (13) and these substrates can provide a good indication of proteasome function in vivo. The cellular degradation is, however, not only dependent on the proteasome itself, but is also a reflection of the efficiency in recognition and unfolding of these particular substrates. Thus, to elucidate the relative contribution of auxiliary factors and the proteasome itself in the degradation of in vivo substrates, it is most often essential to determine the specific activity of the proteasome alone, often referred to as *proteasome capacity*. However, since the interactions between the 20S proteasome and its different regulators are perturbed or compromised by mutually exclusive extraction conditions (i.e., NaCl favoring the 26S composition but breaking up the interaction between 20S and PA28αβ; 14, 15), we propose a systematic in vitro assay allowing different types of proteasome/regulator interactions that enables the reconstruction of cellular proteolytic processes.

The method presented here describes how to assay the hydrolytic activities of the cytosolic proteasome complexes 26S and PA28-20S in ES cell lysates using a fluorogenic peptide: a nonfluorescent compound that upon enzymatic activity produces fluorescence. The substrate used here, succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC), is a short peptide whose amino acid sequence is recognized by the 20S-subunit β5 (and β5i that replaces β5 in the so-called immunoproteasome, 20Si). β5 exhibits chymotrypsin-like cleavage activity which releases the AMC molecule and since free AMC is fluorescent it can be detected using 380 nm excitation and 460 nm emission filters. The procedure consists of two steps: (1) preparation of crude ES cell cytosolic extracts under conditions that favor the 20S interactions to either 19S or PA28 and (2) proteasome activity measurements assaying either 26S, PA28-20S, or 20S alone (*see* Table 1). The peptide hydrolysis efficiency of each complex is assayed separately and thus in combination gives a qualitative analysis of the overall proteasome peptidase capacity in the cytosol.

2 Materials

Use ultrapure water (typically prepared by purifying deionized water to attain a resistivity of 18.2 MΩ · cm at 25 °C) and analytical grade reagents in the preparation of all solutions.

Table 1
Buffers for lysis and assay of cytosolic proteasomes

Lysis buffers	PA28-20S	25 mM Tris/HCl, pH 7.5 0.2 % NP40
	26S	25 mM Tris/HCl, pH 7.5 0.2 % NP40 100 mM NaCl 5 mM ATP, pH 7.5 20 % Glycerol 0.5 mM DTT (added after BCA assay)
Activity assay buffers	PA28-20S	25 mM Tris/HCl, pH 7.5 0.5 mM DTT
	26S	25 mM Tris/HCl, pH 7.5 5 mM ATP, pH7.5 0.5 mM DTT
	20S	25 mM Tris/HCl, pH 7.5 0.02 % SDS 0.5 mM DTT

2.1 Components for Preparation of Cytosolic Lysates

1. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4. For 1 L, dissolve 8 g of NaCl, 0.2 g of KCl, 1.42 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 800 mL water, adjust pH to 7.4 with HCl, and fill up volume to 1,000 mL with water. Store at room temperature.
2. Cell scrapers.
3. Eppendorf centrifuge 1,000 × *g* (room tempered) and 5,000 × *g* (4 °C).
4. Tris/HCl 1 M, pH 7.5: Dissolve 12.114 g Tris base in 80 mL water, set pH to 7.5 using HCl, and adjust volume to 100 mL with water. Store at room temperature.
5. Nonidet P40 Alternative (NP-40A), 10 % Solution, Sterile-Filtered (Calbiochem).
6. ATP 50 mM, pH 7.5: For 10 mL 50 mM stock dissolve 0.275 g in 8 mL water, set pH to 7.5 with NaOH, fill up to 10 mL with water, and store in 500 μL aliquots at −20 °C. Stable for at least 6 months.
7. Dithiothreitol (DTT) 0.1 M: Dissolve 0.154 g in 10 mL water, and store in 500 μL aliquots at −20 °C. Stable for at least 6 months. Wear appropriate protective equipment during handling.
8. PA28 lysis buffer: 0.2 % NP-40A and 25 mM Tris (pH 7.5). For 10 mL gently mix 200 μL NP-40A (10 %), 250 μL Tris/HCl (1 M, pH 7.5), and 9.6 mL water. Store at 4 °C.

9. 26S lysis buffer: 0.2 % NP-40A, 100 mM NaCl, 5 mM ATP, 20 % glycerol, 25 mM Tris/HCl (pH 7.5), 0.5 mM DTT. For 10 mL gently mix 200 μL NP-40A (10 %), 0.058 g NaCl, 1 mL ATP (50 mM, pH 7.5), 2 mL glycerol (≥99 %, or 2.3 mL 87 %), and 250 μL Tris/HCl (1 M, pH 7.5). Adjust volume to 10 mL with water. Store at 4 °C. Add 0.5 μL DTT (0.1 M) per 100 μL 26S lysis buffer prior to use *if compatible with protein concentration assay*; otherwise add DTT after an aliquot has been set aside for this purpose.
10. Protein concentration determination kit/protocol, for example the BCA Protein Assay kit (Pierce).
11. Microplate Spectrophotometer (for protein concentration determination).
12. 96-Well plates for optical density measurements (for protein concentration determination).

2.2 Components for Proteasome Activity Measurements

1. Sodium dodecyl sulfate (SDS) 2 %: Dissolve 0.02 g SDS in 1 mL water; make fresh at the same day of activity assay.
2. Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC; Bachem): To make 4 mM stock, dissolve 0.0306 g in 10 mL DMSO (wear appropriate protective equipment during handling), and store in 200 μL aliquots at −20 °C. Stable for at least 6 months.
3. Lactacystin (Enzo Life Sciences): To make 1 mM lactacystin, dissolve 200 μg in 0.531 mL H_2O, and store in 75 μL aliquots at −20 °C. Stable for at least 6 months. Wear appropriate protective equipment during handling.
4. 7-Amino-4-methylcoumarin (AMC; Bachem): To make 10 mM stock, dissolve 0.0175 g AMC in 10 mL DMSO (wear appropriate protective equipment during handling), and store in 1 mL and 5 μL aliquots at −20 °C.
5. Fluorometer microplate reader enabling excitation at 340–390 nm λ and detection of emission at 430–470 nm λ and a temperature at 37 °C.
6. 96-Well plates for fluorescence measurements (black most optimal).
7. SDS-PAGE equipment.
8. SDS-PAGE gel 10–12 % acrylamide.
9. Loading dye for Western.
10. Coomassie stain and destain solutions.
11. Quantification software (for Coomassie-stained control gel), for example Fiji (Fiji Is Just ImageJ; National Institutes of Health).

3 Methods

3.1 Preparation of Cytosolic Lysates for Proteasome Activity Measurements

1. Gently wash ES cells twice with PBS. Add 0.5–1 mL PBS and use cell scrapers to detach the cells and transfer them to pre-weighed Eppendorf tubes (*see* **Note 1**).
2. Centrifuge at 1,000 × *g* at room temperature for 5 min and remove as much as possible of supernatant. Weigh the tube again, make a note of the pellet weight (weight of tube and pellet subtracted by the weight of the tube), and if there are samples to be compared that are harvested at different time points, freeze the tubes at −80 °C (*see* **Notes 2** and **3**).
3. Dissolve pellet in 100 μL PA28 or 26S lysis buffer for every 0.01 g of pellet (*see* **Notes 4**–**7**) and incubate gently agitating for 30 min on ice. Remove nuclei and cell debris by centrifugation at 5,000 × *g* and 4 °C for 5 min and transfer the supernatant to a new tube. If the protein concentration assay is incompatible with DTT, set aside an aliquot (20 μL) for determining protein concentration and thereafter add DTT to the 26S lysates.
4. Determine protein concentration of the cytosolic extracts. For the BCA kit procedure follow the kit instructions or do as follows: To a 96-well plate for spectrophotometric analysis, add 10 μL of each concentration of the BSA standard (2,000, 1,000, 500, 250, 125, 62.5 μg/mL in the lysis buffer used), 5 μL lysis buffer in the wells for the samples, 5 μL of samples in these wells, mix BCA reagents 1:50, add 200 mL of BCA reagent solution to each well, and mix by pipetting up and down. Incubate at 37 °C for 30 min and measure absorbance at 562 nm. Determine protein concentration using the function describing the linear curve fit of the BSA standard.

3.2 Proteasome Activity Measurements

1. Have the following solutions for the proteasome activity measurements ready on ice: cytosolic extracts (diluted in 26S/PA28 lysis buffer so that they all have the same protein concentration), ATP (50 mM, pH 7.5), AMC (5 μL stock of 10 mM), DTT (0.1 M), and lactacystin (1 mM). Have Tris/HCl (1 M, pH 7.5), SDS (2 %), and suc-LLVY-AMC (4 mM) ready at room temperature.
2. Prepare the 2× assay solutions. 1.5 mL is sufficient for 28 wells in the 96-plate format (*see* **Note 8**). For the 2× 26S assay solution (**Notes 6** and **7**) mix 37.5 μL Tris/HCl (1 M, pH 7.5), 300 μL ATP (50 mM, pH 7.5), 7.5 μL DTT (0.1 M), and 1.155 mL water. To make the 2× 20S assay solution (**Notes 7** and **9**), mix 37.5 μL Tris/HCl (1 M, pH 7.5), 15 μL 2 % SDS, 7.5 μL DTT (0.1 M), and 1.440 mL water. For the 2× PA28 assay solution, mix

37.5 μL Tris/HCl (1 M, pH 7.5), 7.5 μL DTT (0.1 M), and 1.455 mL water.

3. Make 1.2 mL of a 25 mM Tris and 0.5 mM DTT solution: 30 μL Tris/HCl (1 M, pH 7.5), 6 μL DTT (0.1 M), and 1.164 mL water. Prepare AMC by adding 495 μL 25 mM Tris and 0.5 mM DTT solution to a 5 μL aliquot.
4. Add the 2× assay solutions, 50 μL to each sample well, followed by 3 μL of lactacystin (1 mM stock, 30 μM in the assay, *see* **Note 10**), cell extracts (same amount in each well, 20–50 μg total protein), and free AMC. To make a reference of free AMC signal pipet 5, 10, 20, 35, 50, 75, and 100 μL for 5, 10, 20, 35, 50, 75, and 100 μM AMC standard. Fill up the AMC standard wells with 25 mM Tris and 0.5 mM DTT solution to 100 μL and the sample wells with water to 95 μL.
5. When all is set for adding the substrate, incubate the plate in the fluorometer at 37 °C for 5–10 min, add the substrate (5 μL 4 mM stock, 200 μM in the assay; *see* **Note 11**) to all wells except those containing the AMC standard, and start measuring AMC fluorescence without delay. Use a program that measures every 2–5 min for 2 h or more.
6. Run SDS-PAGE gel with the same relative amount of the protein extracts as used in the assay scaled down to approximately 10 μg. Coomassie stain the gel and if the protein levels appear to differ by eye, scan the gel and quantify the intensity of the lanes using a quantification software.
7. Calculate the slope of the increase in AMC fluorescence (in mol AMC extracted from the AMC standard curve) from each well by trend line equation of the area with the highest slope (same time interval for all wells). Divide by μg total protein (if differing in step 6, normalize accordingly) and present proteasome activity results as specific activity (ρmol of free AMC/min and μg total protein).

4 Notes

1. Weigh the empty Eppendorf tubes individually since they vary in weight.
2. When comparing enzymatic activities of different samples it is important to make these samples as similar as possible except for the variable that is put to test in the analysis. We have found that the best way to minimize variations in the treatments of the samples when taken at different time points (days) is to weigh and freeze the cells just after sampling. Doing so, the samples are relatively well preserved as whole cells at −80 °C; they can be lysed at the same time—just before analysis—and at the same

level of molecular crowding, as the volume of lysis buffer can be adjusted to the amount of cell material.

3. Freezing is not necessary if the time of sampling is only differing with a couple of hours and lysis and assay can take place thereafter. If so, leave the pellet on ice instead of freezing it and proceed with the protocol when you have all the samples to be assayed so that they can be run simultaneously.
4. Nonidet P40 Alternative (NP-40A) is a nonionic detergent that breaks up the cell membrane without affecting 20S-regulator interactions, and the lysis procedure described here does not destroy the nuclear membrane (16).
5. The PA28-20S interaction is disrupted by 100 mM NaCl while this salt concentration is beneficial for 26S (14, 15). Intracellular concentration of Na^+ in mammalian cells is around 12 mM and Cl^- 4 mM (17); there are lysis protocols using the more physiological 10 mM NaCl (e.g., (18)) or 5 mM $MgCl_2$ (19). Assays done on these lysates may give a good indication of total proteasome activity, though no comparative studies of the effects of Na^+ and Cl^- concentrations in this range have been done. Using +/−100 mM NaCl in the lysis buffers we can distinguish between activities from different proteasome complexes and will thus cover a greater scope of proteasome capacity (20).
6. NaCl, glycerol, and ATP are important to maintain the interaction between 20S and 19S in cytosolic lysates; to assay the ATP-dependent 26S activity, ATP needs to be present (15).
7. DTT is a reducing agent important to include since at least 26S (21) and 20S are susceptible to oxidation (22).
8. Using the 96-well plates, a well plan in Excel is quite handy. Here all the wells and what will be added in them can be noted. Doing so makes it easy to calculate how much of each component is required in the assay and to keep track during pipetting.
9. 0.02 % SDS is used in the 20S assay buffer to dissociate 19S from 20S and to activate 20S peptidase activity (15).
10. Lactacystin needs to be modified to clasto-lactacystin β-lactone to inhibit proteasome function (23), and since lactacystin inhibits suc-LLVY-AMC cleavage to 90 % in the lysates from mouse ES cells prepared as described here (20) this modification does take place. Using other cell types this may not be the case. Then we recommend using 500 nM of the inhibitor epoxomicin which is also a quite specific inhibitor of the chymotrypsin-like proteasome activity (24). For routine analysis of known samples, the less expensive but relatively unspecific MG132 could be used, but this inhibitor is not recommended to be used as the only proteasome inhibitor. Moreover, it is

important to know that both lactacystin and epoxomicin can also inhibit some lysosomal cathepsins. One way to lower the influence of these is to increase the pH to 8.3. There are also some cytoskeleton-localized calpains (not inhibited by epoxomicin; 25) that exhibit chymotrypsin-like activity. Since the proteasome is an unusually large protease, some kind of size discrimination in combination with activity measurements is necessary to ensure that it is proteasomes and not other proteolytic enzymes that are being assayed. Examples of this approach include assaying fractions from gel filtration (5, 15), assaying proteasome complexes separated by native gel (26), or using a 500 kDa Mw cutoff membrane (27).

11. Suc-LLVY-AMC assays the chymotrypsin-like activity, i.e., cleavage after hydrophobic amino acids, which is carried out by β5 subunits in 20S and β5i subunits in 20Si. This activity is readily inhibited by the specific proteasome inhibitors lactacystin and epoxomicin and is thus easily verified. It is also possible to use other substrates to assay the β1/β1i peptidylglutamyl-peptide hydrolyzing (PGPH, or caspase-like) activity or the β2/β2i trypsin-like activity of the 20S proteasome (19, 27). However, the interpretation of the results is complicated since PGPH activity is not inhibited by lactacystin or epoxomicin and the tryptic activity is not specific for the proteasome (27).

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A Concise Protocol for siRNA-Mediated Gene Suppression in Human Embryonic Stem Cells

Peter F. Renz and Tobias A. Beyer

Abstract

Human embryonic stem cells hold great promise for future biomedical applications such as disease modeling and regenerative medicine. However, these cells are notoriously difficult to culture and are refractory to common means of genetic manipulation, thereby limiting their range of applications. In this protocol, we present an easy and robust method of gene repression in human embryonic stem cells using lipofection of small interfering RNA (siRNA).

Keywords: Transfection, Human embryonic stem cells, Lipofection, siRNA mediated-knockdown, Gene silencing

1 Introduction

Ever since the isolation of human embryonic stem cells (hESCs) from the inner cell mass of the blastocyst and the discovery of their unlimited potential for self-renewal (1) they spawned their own field of biological research. Their ability to differentiate into virtually any cell of the body makes them an invaluable tool for disease modeling. Furthermore, recent publications indicate that they are becoming a powerful therapeutic tool for regenerative medicine (2, 3). In addition, they can serve as a model for early human embryonic development, a process difficult to study due to ethnical concerns. Thus, understanding the molecular mechanisms governing self-renewal and differentiation is a critical goal of today's research.

However, maintenance of embryonic stem cells in vitro has proven to be labor intensive and genetic manipulations are even more difficult to achieve compared to many other cell lines (4). Classical gene disruption strategies to study loss of function in hESCs are tedious and time consuming (5). Limitations arise mostly by the resistance of hESCs to exogenous DNA and RNA, low transfection efficiency, or high cytotoxicity of conventional gene transfer methods. A potential cause for low transfection efficiency may lay in the fact that hESCs are passaged as small colonies, thereby

inhibiting efficient transfer of liposomal packed DNA or RNA. However, hESCs show low colony forming potential when seeded as single cells and recent reports suggested an increased risk of genome alteration induced by single cell propagation (6).

Here in this protocol, we present a lipofection-based approach to transfect hESCs with siRNA.

The following transfection method is convenient, reproducible, and highly effective. Combined with the induction of temporary gene silencing mediated by RNA interference it represents a quick alternative to study loss of function in human embryonic stem cells. We exemplified this by suppressing the expression of a key pluripotency factor OCT4 (POU5F1) using siRNA. These small RNA target OCT4 mRNA and inhibit its translation or mRNA degradation leading to a downregulation at the protein level.

2 Materials

Opti-MEM® (Life Technologies).

Lipofectamine® RNAiMAX Reagent (Life Technologies).

Accutase® (STEMCELL Technologies).

ReLeSR™ (STEMCELL Technologies).

PBS 1× without Mg^{2+}/Ca^{2+} (Life Technologies).

Matrigel, hESCs tested (BD Bioscience/Corning).

TRIzol® (Life Technologies).

Superscript Reverse transcriptase II (Life Technologies).

Rabbit anti-OCT4 (Santa Cruz, sc-9081).

Rabbit anti-GAPDH (Sigma, G9545).

Primers and siRNA used are listed in Table 2.

3 Methods

In the first part of the protocol, we present the basic culture condition for hESCs followed by a detailed description of the transfection procedure (outlined in Fig. 1). In the last part, we briefly introduce the procedure to evaluate the gene suppression efficiency by RT-qPCR and immunoblotting (Fig. 2).

3.1 Maintenance of hESCs

hESCs (WA09, WiCell) were maintained in mTeSR1 or in murine embryonic fibroblast conditioned medium respectively (*see* **Notes 1** and **2**). Medium compositions are listed in Table 1. Experiments with hESCs were approved by the local authorities. (Permission number: R-FP-S-1-0008-0000)

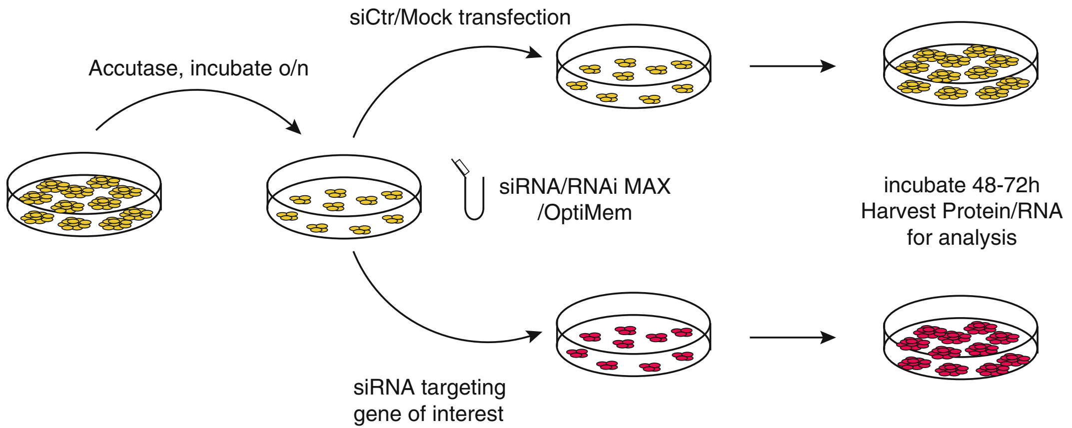

Fig. 1 Schematic representation of the experiment

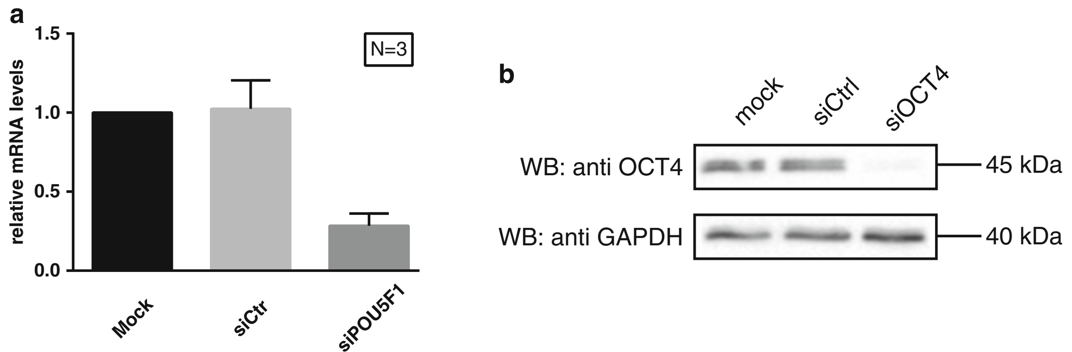

Fig. 2 Efficient transfection of human ESCs with siRNA targeting OCT4/POU5F1. (**a**) H9 hESCs were transfected as described and total RNA isolated 48 h after transfection. The expression level of *OCT4*/*POU5F1* mRNA was assessed by RT-qPCR and normalized to *GAPDH* and *HPRT*. *Bars* represent means +/− SD ($N = 3$) (**b**) OCT4/POU5F1 protein levels were assessed 48 h after siRNA transfection by immunoblot. GAPDH protein levels serve as loading control. Representative blots of three independent experiments are shown

Carry out all procedures in a sterile hood at room temperature unless specified otherwise.

Production of MEF conditioned medium:

1. 2.4×10^6 mitomycin treated MEFs were plated on a 15 cm gelatin (0.2 % w/v) coated dish in MEF medium and were cultured overnight at 37 °C/5 %CO_2 in a tissue culture incubator.
2. The MEFs were washed twice with PBS and the MEF medium was replaced with 30 ml of H9 medium per 15 cm dish. After 1 day, the conditioned H9 medium was collected, sterile filtrated and stored at 4 °C. The medium was replenished and collected after an additional incubation of 24 h.

Table 1
Mediums used

	Components	Volume
MEF medium:	DMEM (Life Technologies, 41965)	500 ml
	Penicillin/Streptomycin (Life Technologies, 15140)	5.5 ml
	Fetal bovine serum	50 ml
H9 medium	DMEM/F12 (Life Technologies, 11330)	500 ml
	Knockout serum replacement (Life Technologies, 10828)	125 ml
	Non-Essential amino acids (Life Technologies, 111409)	6.125 ml
	Penicillin/Streptomycin (Life Technologies, 15140)	6.125 ml
	β-mercaptoethanol	0.1 mM (final)
	L-Glutamine(Life Technologies, 15090)	6.125 ml
	FGF2 (Peprotech)	4 ng/ml (final)
mTeSR1	mTeSR1 (STEMCELL Technologies)	500 ml
	Penicillin/Streptomycin (Life Technologies, 15140)	5 ml

3. The MEFs were discarded after collecting MEF conditioned medium for up to 7 days.

Important: *Re-supplement the conditioned medium with fresh FGF2 (4 ng/ml) prior to use on hESCs.*

Preparation of Matrigel-coated plates:

1. The Matrigel was thawed on ice in the fridge overnight and diluted according to the manufacturer's instructions.
2. Precooled 6- or 12-well plates (Falcon) were coated with ice-cold Matrigel overnight at 4 °C (0.5 ml per 12 well/1 ml per 6 well).
3. Prior to use, the plates were incubated at room temperature for 1 h to solidify the Matrigel. The Matrigel was aspirated and the coated plates were covered with 0.5 (12 well) or 1 ml (6 well) medium.

Passaging of H9 cultured in MEF conditioned medium:
H9 cells were passaged when individual colonies started to touch (5–7 days after passage).

1. H9 cells were washed twice with PBS (2 ml per 6 well).
2. 1 ml of collagenase IV (1 mg/ml, STEMCELL Technologies) was added per 6 well and incubated at 37 °C for 5–10 min (Check whether edges of colonies start to lift up).
3. The cells were washed twice with DMEM/F12 (*Careful not to detach the colonies!*).
4. Cells were gently scraped with a p1000 pipette tip into 1 ml of H9 medium and collected in a 15 ml conical tube.
5. Repeat **step 4** once and pool the cell clumps.

Table 2
Primers and siRNA used

siRNA			
Gene_Symbol	*Product no.*	*Sequence*	Remarks:
POU5F1/OCT4	M-019591-03-0005	(1) 5′ GCGAUCAAGCAGCGACUAU 3′ (2) 5′ GCUCGCAGACCUACAUGAA 3′ (3) 5′ UCAUGAAGAAGGAUAAGUA 3′ (4) 5′ GCUCUUGGCUCCAUGGGUU 3′	(Dharmacon)
siCtr	Custom made	5′ GGGCAAGACGAGCGGGAAG 3′	(Dharmacon) (8)
qPCR primers			
Gene_symbol	*Accession no.*	*Sequence forward*	*Sequence reverse*
GAPDH	NM_002046.3	5′AATCCCATCACCATCTTCCA3′	5′TGGACTCCACGACGTACTCA3′
HPRT	NM_000194.2	5′TCCAAAGATGGTCAAGGTCGCAAG3′	5′TGGCGATGTCAATAGGACTCCAGA3′
OCT4/POU5F1	NM_203289.4	5′TGGGTGGAGGAAGCTGACAACAAT3′	5′TTCGGGCACTGCAGGAACAAATTC3′

6. Add 4 ml of H9 medium and distribute 1 ml of cell suspension on Matrigel-coated dishes containing 1 ml of medium per 6 well. This results in a 1:6 split ratio.
7. The medium was changed every day.

Passaging of H9 cultured in mTeSR1:
H9 cells were passaged when individual colonies started to touch (4–6 days after passage)

1. H9 cells were washed twice with PBS (2 ml per 6 well).
2. 1 ml of ReLeSR (STEMCELL Technologies) was added per 6 well and 0.8 ml was removed immediately. The cells were incubated at 37 °C for 5 min.
3. 1 ml of mTeSR1 was added to the cells and the plate was gently rocked to detach the cells. The cells clumps were collected in a 15 ml conical tube.
4. Repeat **step 3** once and pool the cell clumps.
5. Add 7 ml of mTeSR1 and distribute 1 ml of cell suspension on Matrigel-coated dishes containing 1 ml of medium per 6 well. This results in a 1:9 split ratio.
6. The medium was changed every day.

3.2 siRNA Transfection

Carry out all procedures in a sterile hood at room temperature unless specified otherwise.

1. 12–16 h prior to transfection: Split confluent hESC grown in mTeSR1 or MEF conditioned medium on Matrigel at a ratio of 1:9 using Accutase® with the following protocol: (*see* **Note 3**).
   – Wash cells twice with PBS 1× without Mg^{2+}/Ca^{2+}.
   – Incubate wells with 1 ml Accutase® per 6 well at 37 °C for 3 min.
   – Add double amount of growth medium to inhibit enzyme activity.
   – Gently swirl plates to detach cells and form aggregates of 10–15 cells.
   – Spin down at 47 × *g* for 3 min, resuspend in mTeSR1 (or MEF conditioned medium) at an appropriate split ratio, and distribute onto a Matrigel-coated 12-well plate.
2. Let cells grow overnight in 1 ml mTeSR™/MEF conditioned medium per well.
3. Prepare the following mix for siRNA transfection for 1 well of a 12-well plate:
   (a) 1.5 μl of siRNA (20 pmol/μl) and 75 μl Opti-MEM.
   (b) 1.5 μl of RNAiMAX in 75 μl Opti-MEM.

4. Incubate separately at room temperature for 5 min.
5. Mix (a) and (b) together and incubate for 20 min at room temperature.
6. Meanwhile replace medium (450 μl per well/12 well plate).
7. Add mixture to cells and incubate for 6 h.
8. Add an additional 1.5 ml medium per well.
9. Change medium early the next day.
10. Harvest cells 2–3 days post transfection for protein or RNA analysis.

3.3 RNA Extraction, qRT-PCR, Protein Extraction, and Western Blot Analyses

Two to three days after transfection, RNA and/or total protein were extracted using standard methods. In brief, cells were lysed in 400 μl TRIzol® and total RNA was purified according the manufacturer instructions. 1 μg of total RNA was reverse transcribed using oligo(dT) primers and SuperScript II Reverse Transcriptase as recommended. Quantitative real-time RT-PCR was performed with gene specific primers (*see* Table 2) and 2×SYBR green master mix (Kappa) using a Roche 480 light cycler. Expression levels of genes of interest relative to *GAPDH* and *HPRT* were calculated using the ΔΔct method (7) (Fig. 2a). For immunoblot analysis, cells were lysed in TNTE lysis buffer supplemented with protease and phosphatase inhibitors as described previously (8). 20 μg of total proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with an anti-OCT4 antibody. To ensure equal loading, membranes were stripped and re-probed with an antibody directed against GAPDH (Fig. 2b).

4 Notes

1. Although not tested, we expect our methods to perform comparably under other feeder-free culture systems such as E8 (9) or the CDM system developed by L. Vallier (10).
2. This protocol was used on H9 cells, but it is expected to work on other hESC lines grown under similar conditions.
3. In addition to Accutase, we successfully used ReLeSR (STEMCELL Technologies) to detach the cells prior to transfection as described above.

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Applying Shear Stress to Pluripotent Stem Cells

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Abstract

Thorough understanding of the effects of shear stress on stem cells is critical for the rationale design of large-scale production of cell-based therapies. This is of growing importance as emerging tissue engineering and regenerative medicine applications drive the need for clinically relevant numbers of both pluripotent stem cells (PSCs) and cells derived from PSCs. Here, we describe the use of a custom parallel plate bioreactor system to impose fluid shear stress on a layer of PSCs adhered to protein-coated glass slides. This system can be useful both for basic science studies in mechanotransduction and as a surrogate model for bioreactors used in large-scale production.

Keywords: Physical microenvironment, Shear stress, Bioreactor, Embryonic stem cells, Induced pluripotent stem cells, Stem cell differentiation, Mechanotransduction

1 Introduction

Emerging tissue engineering and regenerative medicine therapies increase the demand for generating clinically relevant numbers of both pluripotent stem cells (PSCs) and cells derived from PSCs (1–3). Large-scale production of cells utilize bioreactors that maintain a well-mixed system by inducing the relative motion of culture medium to cells, which creates fluid shear stresses at the surfaces of cells. Several recent studies have shown that shear stress can affect pluripotent stem cell expansion and differentiation. Shear stress during PSC expansion has been shown to regulate the gene expression of pluripotent markers (4, 5). Furthermore, the application of shear stress during PSC differentiation promotes general mesodermal specification (6), as well as differentiation toward cardiac (7), hematopoietic (8, 9), and endothelial (10, 11) phenotypes. Given the implications of fluid shear stress on stem cell fate and the complexities and expense associated with large-scale systems, it is important to study the mechanistic effects of this physical cue in smaller, well-characterized systems.

A parallel plate bioreactor system allows the use of well-defined fluid flow profiles to induce shear stresses to cells attached to an adherent surface (10, 12). The shear stress is directly proportional

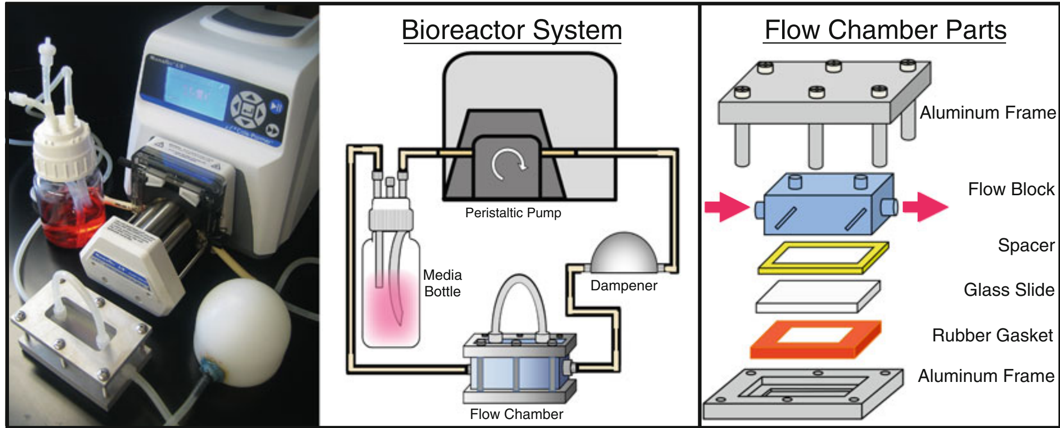

Fig. 1 Bioreactor system. The bioreactor system (*left*) uses a peristaltic pump to recirculate medium from a bottle, through a pulse dampener, across a flow chamber, and back again (schematic in *middle*). The flow chamber is an assembly of a flow block above a spacer that sits on a glass slide, all held together by a top and bottom aluminum frame with a gasket to prevent leaks (schematic in *left*)

to flow rate, which the researcher can control using commercially available low-cost pumps that are usually capable of imposing stresses that range at least an order of magnitude and are relevant to stir-based systems (2, 13). Since these closed-loop systems fit within standard laboratory incubators with CO_2 and temperature regulation, cells can be continuously exposed to shear stress for multiple days. Using such a bioreactor system (*see* Fig. 1), the following is a protocol for differentiating PSCs for 2 days and then exposing them to 2 days of 15 dynes/cm^2 of fluid shear stress, conditions we have used for multiple studies (6, 9, 10). Briefly, PSCs are cultured on a protein-coated glass slide and then placed within a custom assembly of parts that creates a channel above the cells over which fluid flow is directed. This assembly is then connected in series with a medium reservoir, a peristaltic pump, and a pulse dampener to expose the cells to shear stresses induced by steady laminar fluid flow. After treatment, cells can be further cultured, passaged, or assessed for gene and protein expression. While the details below are for a specific protocol with PSCs, various experimental parameters can be changed and alternate cellular phenotypes can be used.

2 Materials

As a protocol that uses a custom bioreactor system, the materials listed below include both commercially available and custom manufactured parts.

2.1 Equipment

1. Chemical fume hood.
2. Laminar flow hood.

3. Autoclave.
4. Vacuum aspiration system.
5. Peristaltic pump (Masterflex, cat. no. 7551).
   (a) 8-channel, 4-roller pump head (Cole-Parmer, cat. no. HV-07519-06).
   (b) Four large cartridges for L/S 25 tubing (Cole-Parmer, cat. no. HV-07519-70).

2.2 General Reagents

1. 10 N sulfuric acid (*see* **Note 1**).
2. 70 % ethanol: 70 ml ethyl alcohol 200 proof to 30 ml of water.
3. Dehydration alcohol.
4. Sodium bicarbonate.

2.3 General Supplies

1. Six acid resistant containers with lids (minimum of 75 mm tall).
2. Slide forceps (EMS, cat. no. 78335-35A).
3. Hemostats.
4. Sterile glass Pasteur pipettes.
5. Absorbent pad with plastic backing (Daigger, cat. no. EF8313AA).
6. 28 mm polyethersulfone 0.2 μm filter (VWR, cat. no. 28200-042).
7. Instant sealing sterilization pouches.
   (a) Small: 5¼ × 10 in. (Fisherbrand, cat. no. 01-812-54).
   (b) Medium: 7½ × 13 in. (Fisherbrand, cat. no. 01-812-55).
   (c) Large: 12 × 18 in. (Fisherbrand, cat. no. 01-812-58).

2.4 Culture Reagents

1. Dulbecco's phosphate buffered saline (DPBS 1×).
2. 50 μg/ml collagen type IV solution: 1 mg collagen type IV (BD Biosciences, cat. no. 354233) in 20 ml 0.05 M HCl (*see* **Note 2**).
3. Culture medium (*see* **Note 3**).
4. Differentiation medium (*see* **Note 4**).

2.5 Culture Supplies

1. 75 × 38 mm micro glass slides (Corning, cat. no. 2947).
2. Disposable cell lifters (Fisherbrand, cat. no. 08-100-240).
3. Plastic dishes.
   (a) 150 mm bacterial culture petri dishes (Corning, cat. no. 430597).
   (b) 100 × 15 mm square sterile Petri dishes with lid (Thermo Scientific, cat. no. 4021).
4. 10 × 13 in. metal trays.

2.6 Bioreactor Loop

1. Tubing.
   (a) Norprene Masterflex L/S 25 tubing (Cole Palmer, cat. no. 06402-25).
   (b) Silicone tubing 0.125″ ID, 0.250″ OD (Cole Palmer, cat. no. 06411-67).
2. Polypropylene luers (all from Value Plastics).
   (a) Male luer integral lock ring to 200 series barb, 3/16″ (cat. no. MTLL250-6).
   (b) Female luer thread style to 200 series barb, 1/8″ (cat. no. FTLL230-6).
   (c) Female luer thread style coupler (cat. no. FTLLC-6).
   (d) Female luer thread style with 5/16″ Hex to 1/4-28 UNF Thread (SFTLL-6).
3. Pulse dampener, 190 ml dead vol, max 60 PSI (Cole-Parmer, cat. no. EW-07596-20).
4. Medium bottle: Nalgene PC 250 ml (Cole-Parmer, cat. no. EW-00091-HX).
5. Medium bottle cap: Nalgene filling/venting cap for 1/4″ tubing (Cole-Parmer, cat. no. EW-06258-10).

2.7 Bioreactor Flow Chamber

1. The parallel plate chamber consists of multiple custom machined parts (*see* Fig. 2a).
   (a) Top aluminum frame.
   (b) Bottom aluminum frame.
   (c) Flow block.
2. Additional parts are cut from stock materials (*see* Fig. 2b).
   (a) Rubber gasket: FDA silicon rubber, 1/8″ thick, 50 A durometer (McMaster, cat. no. 86045K13).
   (b) Spacer: shim, 0.020″ (~0.5 mm) thickness (Precision Brand, cat. no. 44160).
3. Philips truss 2″ machine screws (McMaster-Carr, cat. no. 91770A205).
4. Philips screwdriver.

3 Methods

3.1 Initial Preparation of Individual Bioreactor Pieces

1. Further customization of the flow block:
   (a) Create a smooth bottom surface by sanding under running water for 5 min with #220 grit paper followed by an additional 5 min with #600 grit paper.
   (b) Teflon tape should be wrapped around female luers and attached to the four ports (two on the side and two on top).

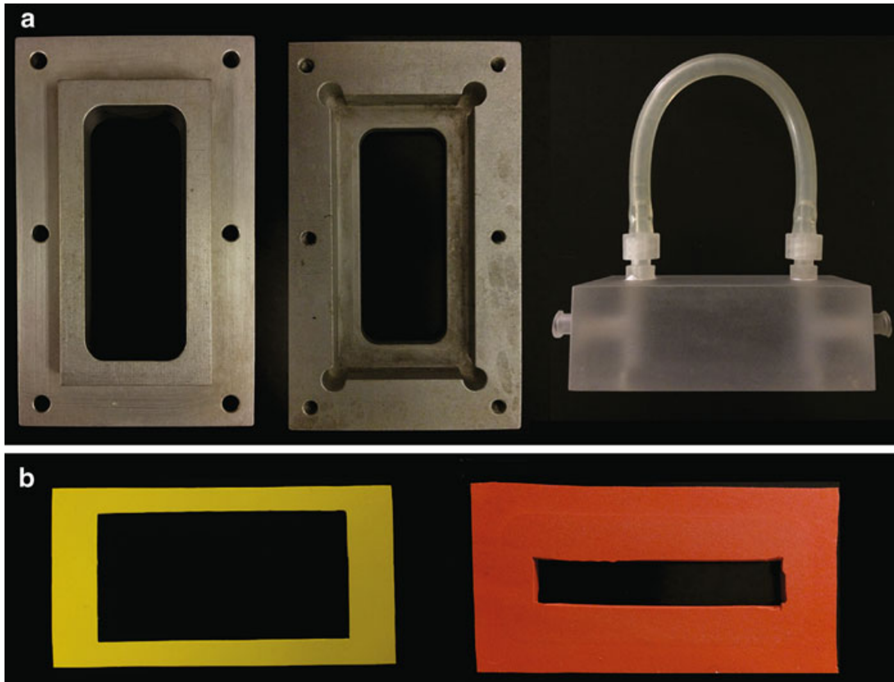

Fig. 2 Pictures of flow chamber components. (**a**) The custom machine parts include (from *left* to *right*) a top aluminum frame, a bottom aluminum frame, and a flow block. (**b**) The items cut from stock materials include (*left*) the spacer, for which the cut width is b and the thickness is h in the shear stress calculations, and (*right*) the gasket, with a slit in the middle to act as a relief that helps cracking of the glass slide

(c) A 7″ piece of silicon tubing should be capped with male luers on each end and then fastened to the top ports of the flow block (*see* **Note 5**).

2. The medium bottle cap needs to be modified to include tubing to the top ports. Silicon tubing approximately 27″ in length should be capped with luers and press-fit onto the cap top to create a source channel (male luer), a return channel (female luer), and a venting port (male luer).
3. The dampener needs to be customized by attaching one set of the provided barbs to the ports on the side per the manufacturer's instructions. The selected barb size should be one that tightly fits the silicon tubing. Attach 5″ pieces of silicon tubing to each, capping the other end of the tubing with a male luer on the other end (*see* **Note 6**).
4. The spacer needs to be cut to have an inner channel with a width of 2.65 cm. The length of the channel needs to span across the slits on the flow block when aligned with the spacer.
5. A relief should be cut (size not critical) in the middle of the gasket to help avoid cracking the glass slide from the pressure of the screws.

3.2 Acid Washing of Slides

1. These steps need to be performed in chemical fume hood using standard laboratory safety procedures.
2. Partially fill three acid resistant containers with 10 N sulfuric acid, two containers with 70 % ethanol, and one container with dehydration alcohol. Allow enough space so that there is no overflow when a glass slide is put into the container.
3. Using slide forceps, hold the slide and immerse it in the first container of 10 N sulfuric acid.
4. Allow the slide to soak for approximately 5 s before transferring to the next container.
5. After letting the slide sequentially soak in each of the three containers of sulfuric acid, submerge the slide in 70 % ethanol. This will help remove excess acid. Move to a second container of 70 % ethanol for additional washing.
6. Briefly dip the slide into dehydration alcohol before placing the slide in a 150 mm petri dish.
7. Rinse the slides with sterile DPBS and allow slides to fully dry before autoclaving.
8. Place three to five slides in small sterilization pouches (avoid overlapping the slides), seal the pouch, and autoclave (*see* **Note 7**). Eight slides will be needed for a standard trial with four experimental shear samples and four static controls.

3.3 Bioreactor Loop Sterilization

1. Assemble a tubing loop by connecting the luers in the following order:
   - (a) Source channel of cap attached to medium bottle.
   - (b) 20″ section of silicon tubing capped by a female luer on each end.
   - (c) 7″ Masterflex L/S 25 tubing capped by a male luer on each end.
   - (d) 20″ section of silicon tubing capped by a female luer on one end and a male luer on the other end.
   - (e) Dampener.
   - (f) 7″ section of silicon tubing with a female luer on one end and a male luer on the other.
   - (g) Female luer coupler.
   - (h) 27″ section of silicon tubing with a male luer on each end feeding back into the return channel of the medium bottle cap.
2. Tighten all connections.
3. Before placing a bioreactor loop into a large sterilization pouch, unscrew the cap from the bottle to allow airflow during the autoclave cycle. Be sure tubing does not lie across the dampener (*see* **Note 8**).

4. Seal the pouch and autoclave the bioreactor loop. For a single pump trial, four loops are needed.

3.4 Flow Chamber Sterilization

1. Place the rubber gasket inside of the bottom aluminum frame and slide into the medium sterilization pouch.
2. Lay the flow chamber on its side and slide it into the pouch so that the flat surface faces away from the aluminum frame (*see* **Note 8**).
3. Slide the top aluminum frame into the pouch and seal the bag.
4. For a single pump trial, four similarly prepared chambers are needed. Autoclave the flow chambers.
5. Put two metal trays in sterilization pouches, seal, and autoclave.

3.5 Protein Coat Glass Slides

1. Using aseptic technique in a laminar flow hood, use sterile slide forceps to move each sterile glass slide into a separate sterile square dish.
2. Pipette 2 ml of the collagen type IV solution onto each slide to coat at a density of 3.5 μg/cm^2.
3. Use a cell lifter to spread the solution across the entire top of the slides, leveraging capillary action to keep the solution from wicking off the surface (*see* **Note 9**).
4. Eight slides will be needed for a standard trial with four experimental shear samples and four static controls.
5. With the lids on the dishes, allow the slides to sit undisturbed at room temperature for 1 h in the laminar flow hood.

3.6 Seed Cells onto Slides

1. Using standard pluripotent stem cell culture techniques, generate a well-mixed cell suspension of 285,000 cells/ml.
2. Use a vacuum aspiration system and Pasteur pipettes to remove the collagen type IV solution from the glass slides.
3. Coat the surface of the glass slides with 1 ml of the cell suspension solution for an initial seeding density of 10,000 cells/cm^2. Use a cell lifter to help spread the solution across the entire surface of the slides.
4. Cover the petri dishes and allow the cells to attach at room temperature for 1 h in the laminar flow hood.
5. Add 25 ml of pre-warmed (to 37 °C) differentiation medium to each dish and transfer dishes to an incubator set at 37 °C and 5 % CO_2.
6. Allow the cells to differentiate on the slides for 2 days.

3.7 Bioreactor Setup and Pre-warming

1. Lay down absorbent pads within the cell culture incubator to protect from any accidental system leaks.
2. Place the pump, complete with pump head and cassettes, within the incubator. The power cord can connect to either

the built-in power socket available in some incubators or be threaded through the filter port in the back of most incubators. Depending on the incubator configuration, occasionally one or more shelves may need to be removed.

3. In the laminar flow hood, open a sterile bioreactor loop. Attach a filter to the open venting port on the medium bottle cap. Tighten all connections as some may have loosened during autoclaving.
4. Add 125 ml of differentiation medium to the medium bottle and close with the cap.
5. Engage the bioreactor loop with the pump by placing the cassette over the Masterflex tubing section. Push the cassette to the back of the pump head to allow room for the remaining cassettes. Make sure the medium bottle is stable in a standing position and that the tubing and dampener are flat on the incubator shelf.
6. Repeat steps 1–5 for all four loops.
7. Start the pump and set to 48 RPM (*see* **Note 10**).
8. Add 125 ml to each of four 150 mm sterile petri dishes and place in the incubator adjacent to the bioreactor systems.
9. Let the bioreactor loops and medium warm in the incubator for a minimum of 2 h.
10. Sterilize the spacers by placing each in an open petri dish in a closed laminar flow hood with the UV light turned on. Sterilize each side with the UV light for at least 30 min, using sterile slide forceps to flip over the spacer. When complete, transfer all the spacers to a single sterile 150 mm petri dish.

3.8 Application of Steady Laminar Shear Stress

1. Stop the pump. Remove all four closed loops from the incubator and place on a clean lab bench lined with an absorbent pad.
2. Inside the laminar flow hood, place the Phillips screwdriver, the Phillips screws, one pair of autoclaved slide forceps, two pairs of autoclaved hemostats, and two autoclaved metal trays.
3. Open the two autoclaved metal trays. Maintain a sterile surface by opening one metal tray and placing it off to the side. Upon the sterile tray, place the open sterile forceps. Create a clean assembly surface by opening the second metal tray and placing it directly in front of you within easy reach. Place the two open hemostats on the assembly surface.
4. Bring into the laminar flow hood the dish with all the sterile spacers, one autoclaved flow chamber, one previously warmed 150 mm dish with medium, and two square dishes with cultured PSCs on glass slides.
5. Remove the bottom aluminum frame with gasket from the sterilization pouch and place on the clean assembly surface.

6. Using the sterile slide forceps, grab a PSC-seeded glass slide and place on the gasket within the bottom aluminum place. Take care to not disturb the top surface seeded with cells.
7. Grab a sterile spacer and place on the glass slide. Use the internal edges of the bottom aluminum frame to align the space with the slide to ensure minimal contact with cells.
8. Using the tubing loop atop the flow block as a handle, remove the flow block from the sterilization pouch and place atop the spacer. Grab the top aluminum frame and place on the flow block.
9. Using the screwdriver and six Philips screws, tighten the flow chamber assembly. Once the screws engage such that all components are touching, take care to evenly tighten the screws to avoid cracking the slide. Tighten sufficiently to avoid leaks.
10. Move one bioreactor loop into the laminar flow hood and open. Place the tubing so that the section with the female coupler lies across the assembly surface.
11. Clamp a hemostat on the tubing on each side of the female coupler. Remove the female coupler and attach each open end of the tubing to the flow chamber assembly.
12. Return the fully assembled bioreactor system to the incubator and engage with the pump. Remove the hemostats and start the pump (preset to 48 RPM).
13. Monitor the fluid flow to make sure it passes across the glass slide and then clamp the top loop of the flow chamber with a binder clip.
14. Place the remaining (of the two in the laminar flow hood) PSC-seeded glass slide into the 150 mm dish with medium. Place the sample in the incubator adjacent to the bioreactor systems to act as a static control.
15. Repeat steps 4–14 until all the bioreactor systems and static controls have been setup. A standard trial consists of four experimental shear samples and four static controls.

3.9 Remove Samples for Analysis

1. Stop the pump while you remove one of the bioreactor systems, then resume flow to the remaining systems. Place the removed system in the laminar flow hoods if it is necessary for subsequent sterile culture of the cells, else place on the lab bench.
2. Disassemble the bioreactor system by first clamping the tubing on each side of the flow chamber with hemostats. Unscrew the flow block from the tubing. Replace the female coupler to reassemble the bioreactor loop.
3. Use the screwdriver to evenly untighten and remove the screws. Place the top aluminum frame and flow block to the

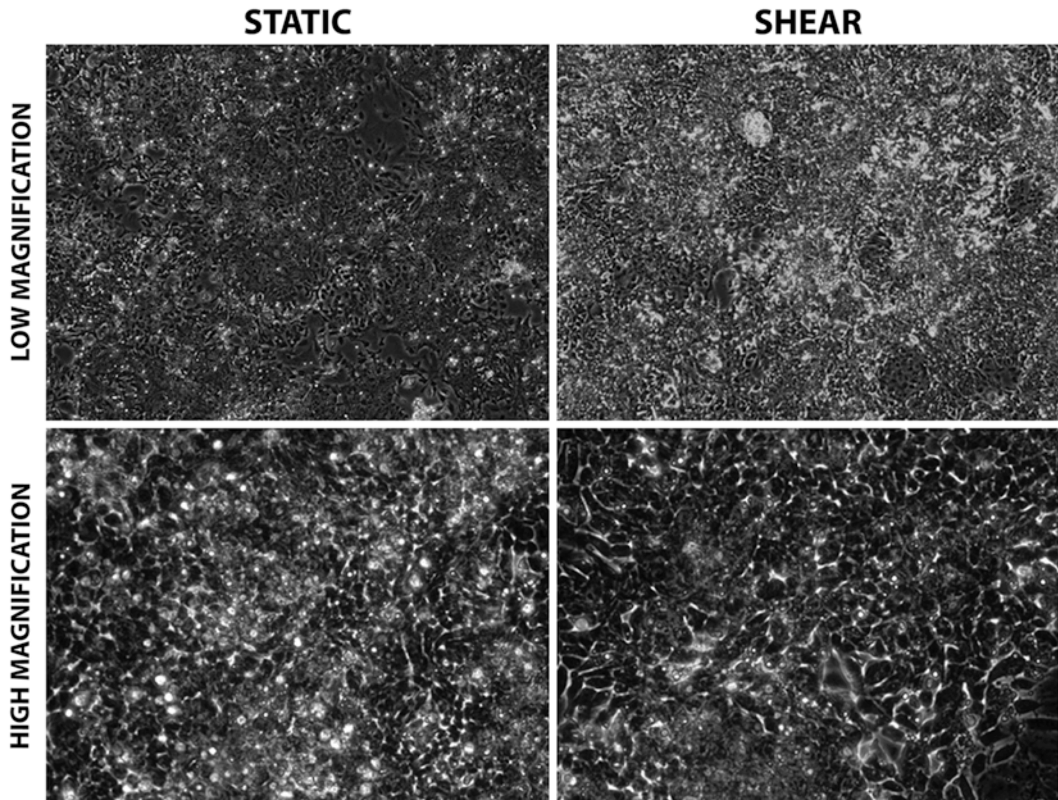

Fig. 3 Phase images of samples. Shown are representative low and high magnification images of pluripotent stem cells from static controls and samples that were exposed to shear stress

side. Remove the spacer with forceps without disrupting the cell layer on the glass slide.

4. Remove the glass slide with cells from the flow chamber assembly. Retrieve one static control sample from the incubator. Trypsinize, lyse, or culture the experimental and control samples using standard cell and molecular biology techniques as appropriate for your experimental design (*see* Fig. 3).
5. Repeat steps 1–4 for the remaining samples.
6. Wash the flow chambers and bioreactor loops with running water before storing.

4 Notes

1. Safe laboratory procedures should always be used. 10 N H_2SO_4 is a strong acid and should be kept in a labeled acid resistant container inside the chemical hood or certified cabinet. To dispose of H_2SO_4, first neutralize with 1.7 g of sodium

bicarbonate ($NaHCO_3$) per gram of acid and then pour into an appropriate waste container.

2. The procedure described here is to differentiate the PSCs for 2 days on collagen type IV and then apply shear stress for 2 days at 15 dynes/cm^2 as previously published (10). In other studies, we have differentiated for 1–3 days on various protein substrates for a range of shear stress magnitudes (6, 9).
3. Various medium formulations can be used to culture PSCs. The formulation used in our lab is currently Dulbecco's Modification of Eagles Medium supplemented with 15 % ES-qualified fetal bovine serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1,000 U/mL Leukemia Inhibitory Factor, and antibiotics.
4. PSCs can be differentiated using a variety of medium formulations, which may be specific to the study. One formulation commonly used in our lab is Minimum Essential Alpha Media supplemented with 10 % fetal bovine serum, 0.1 mM beta-mercaptoethanol, and antibiotics. For a study with four experimental samples and four static controls, 1,200 ml of differentiation medium is needed.
5. The passage of air bubbles over adherent cells can cause cell death. The loop on the top of the flow block acts as a trap for air bubbles, which will rise into the loop. During the bioreactor setup, a binder clip is clamped to the loop to ensure that the medium will flow over the surface of the cells (the path of least resistance).
6. The manufacturer suggests using Teflon tape when securing the barbs to the dampener. In our lab, we apply gasket sealant at the base of the barb for additional protection from leaks.
7. Settings for sterilization of dry items can vary by manufacturer and model of autoclave. Many wall mounted steam autoclaves have a "gravity cycle" of 20 min of sterilization time at 121.0 °C followed by 20 min of drying time, which is appropriate for all the solid bioreactor components except for the spacers.
8. When packing items to be autoclaved, it is important to be aware that contact between certain materials can cause deformation at such high temperatures. During autoclaving for this protocol, it is important that the aluminum frames should not contact the bottom of the flow block and the Masterflex tubing should not contact the dampener.
9. When the slides are being coated with proteins or seeded with cells, it is important that the solution spreads across the top surface and does not wick over the edges. Otherwise, the slides will not be covered with the target protein concentration or cell density. After protein coating the slides, it is important to

thoroughly aspirate the solution off the surface to help keep the subsequent application of cell solution from wicking.

10. Shear stress for this system is calculated by the formula $\tau = 6\ Q\mu/bh^2$, where b is the channel width, h is the channel height, μ is the viscosity of the medium, and Q is the fluid flow rate. The values for b and h are set by the dimensions of the spacer and in our system are 2.65 and 0.05 cm, respectively. The viscosity for our differentiation medium formulation is 0.012 dynes × s/cm^2. The flow rate Q is set by the pump, which at 48 rpm results in 15 dynes/cm^2 of fluid shear stress for the parameters in this protocol.

Acknowledgement

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Generating Inner Ear Organoids from Mouse Embryonic Stem Cells

Emma Longworth-Mills, Karl R. Koehler, and Eri Hashino

Abstract

This protocol describes a three-dimensional culture method for generating inner ear sensory epithelia, which comprises sensory hair cells and a concurrently arising neuronal population. Mouse embryonic stem cells are initially plated in 96-well plates with differentiation media; following aggregation, Matrigel is added in order to promote epithelialization. A series of small molecule applications is then used over the first 14 days of culture to guide differentiation towards an otic lineage. After 16–20 days, vesicles containing inner ear sensory hair cells and supporting cells arise from the cultured aggregates. Aggregates may be analyzed using immunohistochemistry and electrophysiology techniques. This system serves as a simple and relatively inexpensive in vitro model of inner ear development.

Keywords: Cell differentiation, Inner ear, Hair cell, Vestibular, Neurogenesis, Cell culture techniques, Three-dimensional cell culture, Stem cell

1 Introduction

During development, formation of the cranial sensory ganglia occurs as a result of a series of signaling cues that guide definitive ectoderm to the formation of non-neural ectoderm and a subsequent pre-placodal epithelium. This pre-placodal region has the capacity to give rise to six neurogenic placodes, from which many structures of the peripheral nervous system are derived. The placode of particular interest here is the otic placode, from which the entire inner ear develops. The otic placode is derived from a region common with the epibranchial placode known as the otic-epibranchial placode domain (OEPD). After induction, the otic placode invaginates to form the otic pit. This pit eventually pinches off from the overlying ectoderm to form the otic vesicle, or otocyst.

The protocol described here aims to illustrate these aspects of inner ear development in vitro, from the induction of non-neural and pre-placodal ectoderm to the formation of the otic placode and subsequent derivation of otic vesicles containing inner ear sensory epithelia (Fig. 1). Mouse pluripotent stem cells are employed in a three-dimensional culture system to recapitulate this differentiation process.

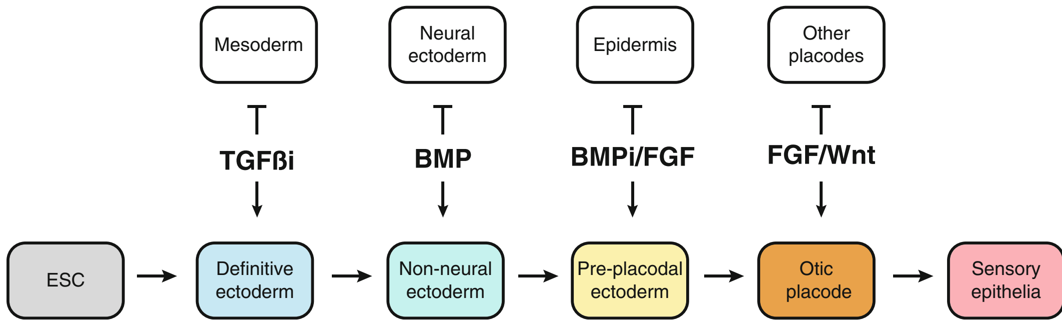

Fig. 1 Schematic of inner ear development, highlighting key signaling mechanisms. This protocol takes advantage of key signaling mechanisms in order to guide definitive ectoderm to an inner ear-like sensory fate. A TGF-β inhibitor blocks formation of mesoderm/endoderm and promotes induction of definitive ectoderm. BMP signaling is used to induce non-neural ectoderm. BMP inhibition and FGF activation are required for pre-placodal ectoderm induction. Subsequent FGF signaling and endogenous Wnt signaling guides further development of otic placodes, otic vesicles and inner ear sensory epithelia

Three-dimensional cell culture systems have been previously used to effectively generate complex epithelia, including the generation of the optic cup, mouse and human cortical tissue, and intestinal tissue (1–7). In traditional monolayer (2D) culture, cell growth is restricted to the culture plate, which ultimately affects the shape and development of the cell population. Three-dimensional or "floating" cell culture systems offer the advantage of freedom of growth; this promotes cellular interactions that allow for self-organization that more closely recapitulates an in vivo environment. This is particularly useful in pluripotent stem cell culture, wherein an aggregate of cells may be manipulated with signaling molecules in order to guide differentiation.

In this protocol, mouse embryonic stem cells are initially aggregated in U-bottom 96-well plates (Fig. 2). Matrigel, which contains extracellular matrix proteins, is applied on day 1 of the culture protocol in order to stimulate the development of a basement membrane around the surface of each aggregate. Shortly after Matrigel addition, the surface layer of cells organizes into an epithelium reminiscent of the definitive ectoderm in the embryo.

Small molecule treatments are then applied on day 3 and 4.5 in order to activate or inhibit several key signaling pathways. Day 3 treatment modulates BMP and TGF-β signaling. BMP signaling has been shown to play an important role in the specification of ectoderm (8–13). Here, BMP signaling is activated in order to promote induction of non-neural ectoderm. Since TGF-β signaling has been shown to promote the induction of mesoderm and endoderm (14–16), SB-431542, a TGF-β inhibitor, is applied to block mesoderm and endoderm formation.

It is important to note that BMP signaling, while essential for non-neural ectoderm induction, needs to be attenuated in order to

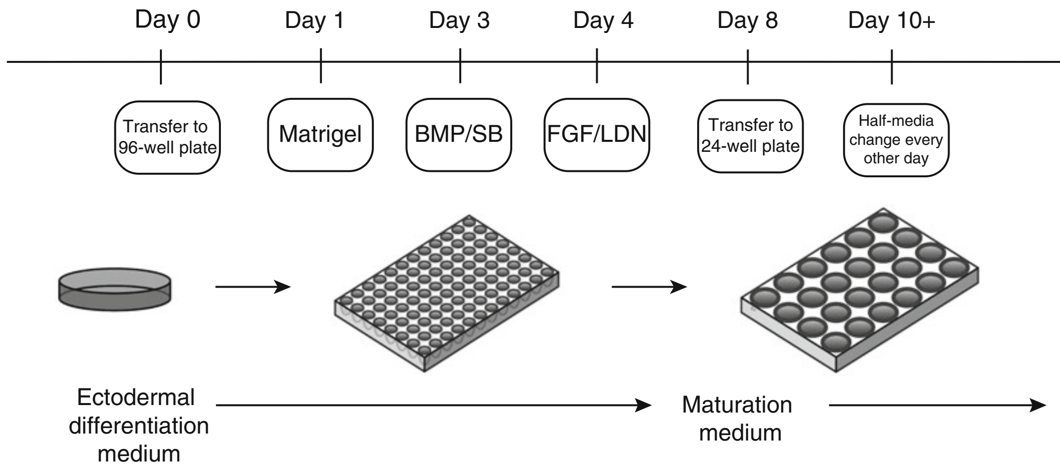

Fig. 2 Schematic overview of the inner ear organoid protocol. Small molecule application and culture conditions are highlighted here. Cells are initially plated in a 96-well plate in Ectodermal differentiation medium. Matrigel and small molecule application proceeds over day 1–4. Transfer to 24-well plate in Maturation medium occurs on day 8, with subsequent half-media changes every other day starting on day 10

guide induction of pre-placodal epithelia (12, 17–20). In this system, LDN-193189 serves as a BMP inhibitor. FGF signaling has been shown to be critical for pre-placodal induction (12, 18–20). The co-application of FGF and LDN-193189 on day 4.5 promotes induction of pre-placodal epithelia. FGF signaling further plays a role in specification of the otic-epibranchial placode domain (21, 22).

On day 8, cultured aggregates are transferred from 96-well to 24-well plates. From here, endogenous Wnt signaling guides induction of the otic placode, and subsequent formation of the otic vesicle (23–25). Morphological cues can be used to track the progression of the aggregates (Fig. 3). From day 6 to 8, aggregates typically display a thickening of the outer epithelium, indicative of OEPD formation. From day 8 to 11, the surface of each aggregate appears to “smooth,” as the cells in the interior of the aggregate migrate out to the surface where they surround the outer epithelium. The first sign of vesicle formation should be apparent on day 12. Typically, vesicles are visible along the outer edges of the aggregates, or towards the interior. These vesicles may stay embedded in the aggregate, or may breach the surface epithelium and protrude from the aggregate (days 14–30). It is within these otic vesicles that the sensory hair cells develop. We designate these hair cell-containing vesicles as inner ear organoids (26).

Interestingly, we have observed the development of a neuronal population in the culture coinciding with inner ear organoid development (26). Our previous evidence suggests that these neurons

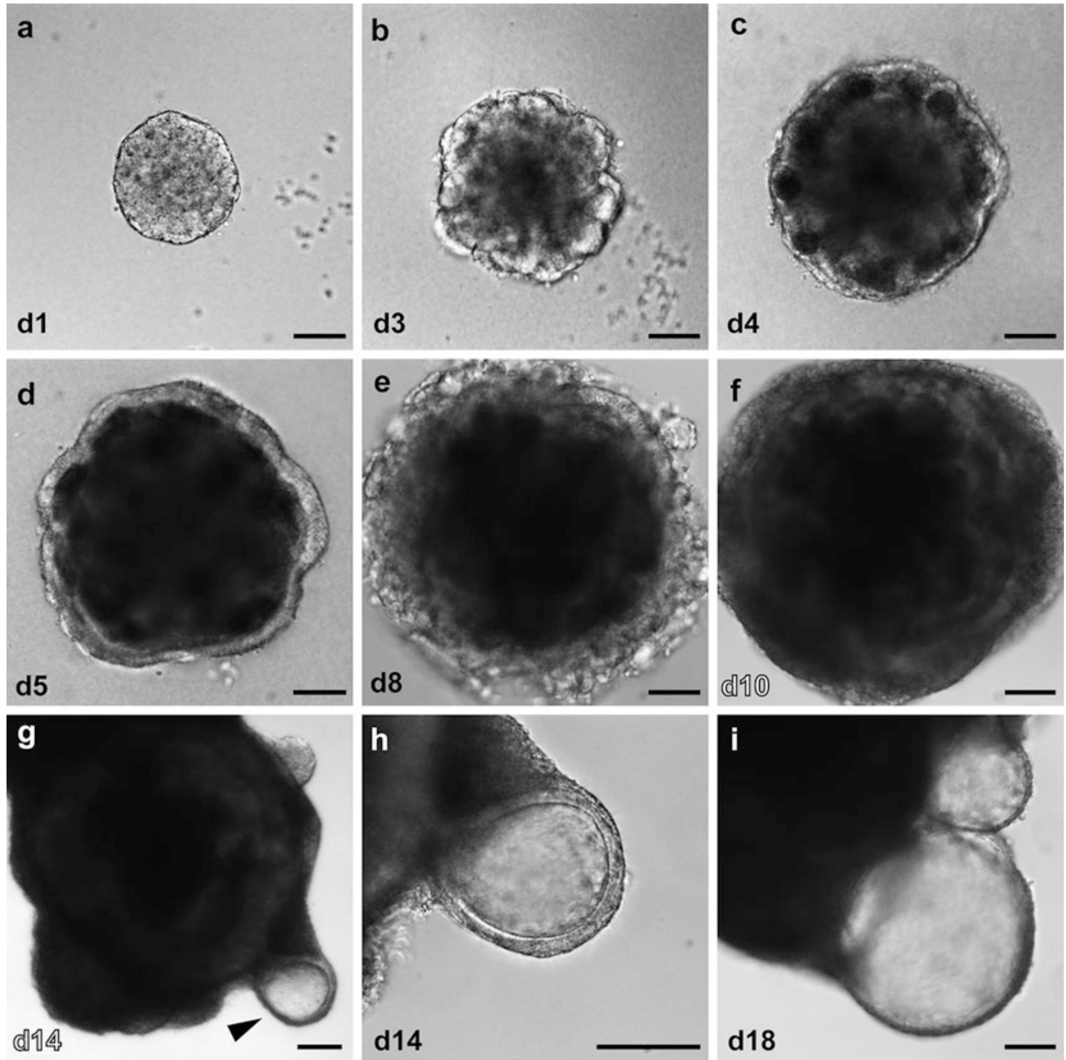

Fig. 3 Aggregate morphology during inner ear organoid culture. DIC images captured from ESC otic differentiation cultures highlight the morphological progression. (**a**–**f**), Early morphology from day 1 to 10. (**g**–**h**) Later morphology; day 14 aggregate, *arrowhead* indicating vesicle formation. (**i**) Day 18 vesicle; site of inner ear sensory epithelia formation. Scale bars, 100 μm

form synapse-like structures with sensory hair cells in the derived inner ear epithelia. Further analysis, however, is necessary to confirm the identity of these sensory-like neurons and whether the synapses are functional.

Aggregates may be fixed and analyzed at any point during the culture period. It may be helpful to fix and analyze aggregates at early time-points to ensure that differentiation is taking place along the desired lineage. We suggest performing immunohistochemical

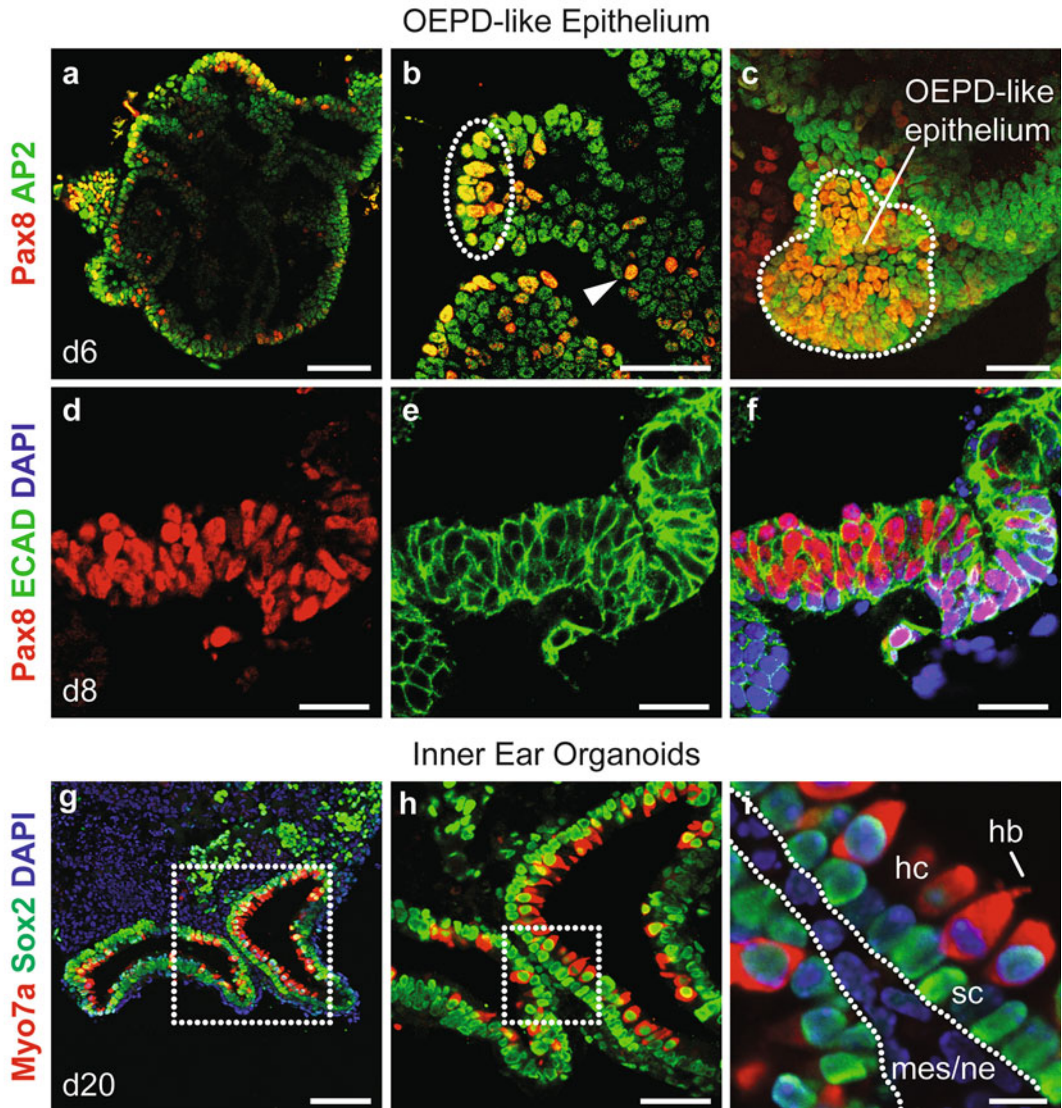

Fig. 4 Immunohistochemistry of OEPD-like epithelium on day 6/8 and inner ear organoids on day 20. (**a**–**c**) Whole-mount staining for Pax8 and AP2 on day 6 reveals patches of Pax8^{+} AP2^{+} cells (*dotted outline*) reminiscent of placodes. Individual Pax8^{+} AP2^{+} (*arrowhead*) cells were observed throughout the epithelium. *Panel* **c** contains a *z*-projection through a representative Pax8^{+} AP2^{+} patch. (**d**–**f**) Representative Pax8^{+} E-cadherin^{+} epithelium on day 8. (**g**–**i**) Protruding inner ear organoids with Sox2+ supporting cells (sc) and Sox2+ Myo7a + hair cells (hc) on day 20. A hair bundle (hb) extends from the apical end of the hair cell in *panel* **i**. Mesenchymal and neural cells surround the inner ear organoids (mes/ne). Scale bars, 100 μm (**a**, **g**), 50 μm (**b**, **c**, **h**), 25 μm (**d**–**f**), 10 μm (**i**)

analysis using antibodies for markers of the OEPD and inner ear sensory epithelia to characterize the derived tissue (Fig. 4).

In summary, this protocol presents an in vitro model for the derivation of inner ear sensory epithelia. Protein and small

molecule treatments are used to guide mouse embryonic stem cell differentiation towards an otic lineage. The resulting tissue contains vestibular hair cells as well as a neuronal population that has been shown to innervate the derived epithelia (26).

2 Materials

2.1 Reagents

1. Mouse ES cells: Prior to use, cells should be acclimated to growth in LIF-2i medium and 80 % confluent. Any well-established mouse pluripotent cell line is suitable for use with this protocol. Although we primarily use a feeder-free ES cell line derived from R1 mice, this protocol has been performed with a mouse iPS cell line and several transgenic ES cell lines, yielding comparable results.
2. GMEM (Gibco, cat. no. 11710–035).
3. Advanced DMEM/F12 (Gibco, cat. no. 12634–010).
4. Sodium Pyruvate, 100 mM (STEMCELL Technologies, cat. no. 07000).
5. Non Essential Amino Acids, 10 mM (STEMCELL Technologies, cat. no. 07600).
6. Penicillin–Streptomycin (STEMCELL Technologies, cat. no. 07500).
7. Knockout Serum Replacement (KSR; Gibco, cat. no. 10828–010). Note that KSR is light sensitive. Store in the dark at −20 °C as per the manufacturer's recommendation.
8. 2-Mercaptoethanol (Gibco, cat. no. 21985–023).
9. Normocin (Invivogen, cat. no. ant-nr-1).
10. N2 Supplement (Gibco, cat. no. 17502–048).
11. GlutaMAX (Gibco, cat. no. 35050–079).
12. Matrigel (BD Biosciences cat. no. 354230).
13. Recombinant BMP4 (Stemgent, cat. no. 03–0007).
14. FGF-2 (Peprotech, cat. no. 100-18B).
15. SB-431542 in solution (Stemgent, cat. no. 04-0010-05).
16. LDN-193189 in solution (Stemgent, cat. no. 04-0074-02). Note that LDN-193189 is light sensitive and should be stored in the dark at −20 °C as per the manufacturer's recommendation.
17. Neurobasal Medium (Gibco, cat. no. 21103–049).
18. B27 Supplement minus vitamin A (Gibco, cat. no. 12587–010).

19. PD-0325901 in solution (Stemgent, cat. no. 04-0006-02).
20. CHIR99021 in solution (Stemgent, cat. no. 04-0004-02).
21. 0.1 % Gelatin (STEMCELL Technologies, cat. no. 07903).
22. 0.25 % Trypsin–EDTA (Gibco, cat. no. 25200–072).
23. Phosphate Buffered Saline (PBS; Gibco, cat. no. 10010–023).
24. ESGRO® Leukemia Inhibitory Factor (LIF; Millipore, cat. no. ESG1106).
25. Dimethyl sulfoxide (DMSO; Sigma, cat. no. D8418).
26. Paraformaldehyde (PFA, Electron Microscopy Sciences, cat. no. 15710).
27. Vacuum grease (Fisher Scientific, cat. no. S41718).
28. Normal goat serum (Vector laboratories, cat. no. S-1000).
29. Urea (Sigma, cat. no. U5378).
30. Glycerol (Sigma, cat. no. G5516).
31. Triton X-100 (Sigma, cat. no. T8787).
32. Antibodies.

2.2 Equipment

1. Cell culture dish (60 mm; BD Falcon, cat. no. 353002).
2. Bacterial dish (100 mm; Fisher Scientific, cat. no. 0875712).
3. U-bottom 96-well plate (Lipidure-Coat; Gel Company, cat. no. LCU96).
4. 24-well plate (Lipidure-Coat; Gel Company, cat. no. LCMD24).
5. Safe-Lock centrifuge tubes (1.5 and 2 mL; Eppendorf, cat. no. 022363204, 022363352).
6. Transfer pipets (Fisherbrand, cat. no. 13-711-7M).
7. Polystyrene round bottom centrifuge test tubes with cell strainer tops (5 mL; BD Falcon, cat. no. 352235)
8. Reagent Reservoirs (VistaLab, cat. no. 21-381-27F).
9. Tissue-Tek Cryomolds (Electron Microscopy Sciences, cat. no. 62534-10).
10. Olympus FV1000 multiphoton microscope or equivalent inverted confocal microscope equipped with a long-working distance objective (20–25×).
11. Cover glasses (24 × 50-1 and 25 × 25 or equivalent; Fisher Scientific, cat. no. 12-544-14 and 12-548-C).
12. 15 mm diameter round cover glasses (thickness no. 0; Assistant, cat. no. 01105509).
13. Biosafety cabinet.
14. CO_2 incubator.

15. Hot plate.
16. Water bath.
17. Desiccator.
18. Cryostat.

2.3 Preparation of Small Molecules

1. *Human recombinant BMP4 stock solution* (*100 ng/μL*): 10 μg of BMP in 100 μL of sterile 4 mM HCl. Vortex and spin down the solution in a tabletop centrifuge. Aliquot BMP4 solution at 5 μL per tube. Aliquots can be stored at −20 °C for 6 months or at −80 °C for 1 year.
2. *Human recombinant FGF-2 stock solution* (*200 ng/μL*): 50 μg of FGF-2 in 250 μL of sterile PBS or 5 mM Tris (pH 7.6). Vortex and spin down the solution in a tabletop centrifuge. Aliquot at 6 μL per tube. Aliquots can be store at −20 °C for 6 months or at −80 °C for 1 year.

2.4 Preparation of Media

1. *LIF-2i ES cell maintenance medium*: For 100 mL basal LIF-2i medium, combine 48 mL Advanced DMEM/F12, 48 mL Neurobasal medium, 2 mL B27 supplement, 1 mL N2 supplement, and 1 mL GlutaMAX in a sterile 250 mL bottle. Basal LIF-2i medium can be used for 3–4 weeks. Immediately before use in culture, prepare complete LIF-2i medium by adding 100 μL Penicillin–Streptomycin, 10 μL LIF, 1 μL PD-0325901, and 3 μL CHIR99021 to 10 mL of basal LIF-2i medium. Complete LIF-2i medium can be used for up to 1 week. It is important to note that B27 should not contain vitamin A when used in LIF-2i preparation for ES cell culture (*see* **Note 1**).
2. *Ectodermal differentiation medium*: For 100 mL ectodermal differentiation medium, combine 95.5 mL GMEM, 1.5 mL KSR, 1 mL sodium pyruvate, 1 mL nonessential amino acids, 1 mL Penicillin–Streptomycin, and 180 μL 2-Mercaptoethanol in a sterile 250 mL bottle. Complete ectodermal differentiation medium can be used for the duration of one experiment or up to 1 week. Adjust the volume of the medium as needed for the number of 96-well plates being prepared.
3. *Maturation medium*: For 100 mL maturation medium, combine 97.9 mL Advanced DMEM/F12, 1 mL N2 supplement, 1 mL GlutaMAX, and 100 μL Normocin in a sterile 250 mL bottle. Complete maturation medium can be used for up to 2 weeks (*see* **Note 2**).
4. *ScaleA2 solution*: For 1 L of scaleA2 solution, dissolve 240.24 g (4 M final concentration) of urea powder in 750 mL of Milli-Q water. Add 1 mL of Triton X-100 (0.1 % vol/vol final concentration) and 100 g of glycerol (10 % wt/vol final concentration). Add Milli-Q water to a final of volume of 1 L. The solution can be stored at room temperature for at least 1 year.

3 Methods

3.1 ESC Dissociation and Plating

1. Warm 0.25 % trypsin-EDTA and LIF-2i medium to 37 °C in water bath.
2. In a Biosafety cabinet, add gelatin to completely cover bottom of 60 mm plate for new passage (*see* **Note 3**). Let the plate sit for approximately 20 min; in the meantime, proceed to **step 3**.
3. Aspirate media from ESC plate (cells at ~80 % confluency) and wash one time with 5 mL PBS.
4. Add 500 μL trypsin-EDTA; incubate at RT or in 37 °C incubator for 1–2 min. Agitate the plate to break up cell clumps and confirm via microscope that cells have rounded and detached from the surface of the plate.
5. Add 1 mL LIF-2i medium to plate and transfer to 2 mL microcentrifuge tube.
6. Pipet in microcentrifuge tube to dissociate cell clumps into single-cells, taking care not to introduce air bubbles into the medium.
7. Centrifuge at RT for 2.5 min at 1,400 rpm.
8. While cells are in centrifuge, aspirate gelatin from the new 60 mm plate; let plate sit for at least 5 min to dry.
9. Remove supernatant from cell suspension by aspirating or pouring; avoid disturbing the pellet.
10. Resuspend in 1 mL LIF-2i medium.
11. Split the cells 1:10 for re-plating in approximately 2 days, or 1: 50 for re-plating in approximately 4 days (*see* **Note 4**).

3.2 ES Cell Differentiation

1. Warm ectodermal differentiation medium in 37 °C water bath.
2. Aspirate LIF-2i media from plate of ~80 % confluent ESCs; wash three times with 5 mL PBS (*see* **Note 5**).
3. Add 500 μL of 0.25 % trypsin–EDTA. Incubate at RT or at 37 °C for 1–2 min. Agitate plate to break up cell clumps and confirm via microscope that cells have rounded and detached from the surface of the plate.
4. Add 1 mL warmed ectodermal differentiation medium to plate; transfer cells to a 2 mL microcentrifuge tube.
5. Dissociate cell clumps into single cells by pipetting up and down with a P1000 tip. Centrifuge for 2.5 min at 1,400 rpm to pellet the cells.
6. Remove supernatant by pipetting or aspirating; avoid disturbing the pellet. Resuspend in 1 mL ectodermal differentiation medium.

7. To a cell-strainer-top test tube, forcefully pipet 1 mL fresh ectodermal differentiation medium. This will prime the strainer. Then pipet 1 mL ES cell suspension into the cell strainer, followed by 1 mL fresh ectodermal differentiation medium, for a total of 3 mL in the tube (*see* **Note 6**).
8. With a P1000 tip, pipet the cell suspension to mix. Use a hemacytometer to determine cell concentration.
9. For two 96-well plates, dilute the appropriate volume of cell suspension in 22 mL fresh ectodermal differentiation medium. The desired final concentration is 30,000 cells per mL. Invert to mix (*see* **Note 7**).
10. Transfer the cell suspension to a reservoir. Using a multichannel pipette, dispense 100 μL cell suspension per well into two 96-well plates.
11. Centrifuge the plates at RT for 5 min at 800 rpm.
12. Store the plates in a 37 °C incubator with 5.0 % CO_2.

3.3 Day 1: Addition of Matrigel

1. Add 440 μL Matrigel to 10.56 mL ectodermal differentiation medium for a total of 11 mL complete medium. It is important to keep the Matrigel on ice or at 4 °C immediately prior to mixing, and to add Matrigel to ice-cold medium, as Matrigel will become gelatinous at temperatures above 15 °C. After mixing, hold the medium up to a light source to confirm that the Matrigel has been fully incorporated.
2. Warm ectodermal differentiation medium with Matrigel in 37 °C water bath.
3. Remove 50 μL of medium from each well using a multichannel pipette, making sure to hold the pipette at an angle so as not to disturb the cell aggregate at the bottom of each well.
4. To each well, add 50 μL of ectodermal differentiation medium plus Matrigel and pipette four to six times to mix (*see* **Note 8**).

3.4 Day 3: Addition of BMP4 and SB-431542

1. For each experimental condition, prepare an appropriate amount of ectodermal differentiation medium. For two 96-well plates, 6 mL of medium is typically adequate.
2. For 6 mL medium, add 3 μL BMP4 and 3 μL SB-431542 (0.5 μL per 1 mL). This results in a 5× concentration of BMP4 and SB.
3. To each well, add 25 μL of ectodermal differentiation medium plus BMP/SB. Note there is a final volume of 125 μL and a final concentration of 10 ng/mL BMP4 and 1 μM SB-431542 per well (*see* **Note 9**).

3.5 Day 4.5: Addition of FGF and LDN-193189

1. For each experimental condition, prepare an appropriate amount of ectodermal differentiation medium. For two 96-well plates, 6 mL of medium is typically adequate.
2. For 6 mL, add 4.5 μL of FGF-2 (0.75 μL per 1 mL) and 3.6 μL of LDN-193189 (0.6 μL per 1 mL). This results in FGF-2 and LDN-193189 at a 6× concentration.
3. To each well, add 25 μL of ectodermal differentiation medium plus FGF/LDN. Note there is a final volume of 150 μL and a final concentration of 25 ng/mL FGF-2 and 1 μM LDN-193189 per well (*see* **Note 10**).

3.6 Day 8: Transfer to Long-Term Culture

1. Cut the end of a P1000 pipette tip and transfer the aggregates from each well into a 15 mL conical tube (*see* **Note 11**). Separate different experimental conditions into different 15 mL conical tubes if necessary.
2. Let the aggregates settle to the bottom of the tube; then aspirate the media, taking care not to disturb the aggregates.
3. Wash the aggregates in 5 mL DMEM/F12 (pre-warmed to 37 ° C).
4. Repeat **steps 2** and **3** at least three times.
5. Remove any excess DMEM/F12 and then resuspend the aggregates in maturation medium containing 1 % Matrigel.
6. Using a wide-mouth P1000, transfer the aggregates to a 100 mm bacterial dish. Suspend in a sufficient amount of maturation medium to account for medium spreading out across the surface of the place.
7. Pipet 1–3 aggregates in 1 mL medium into the wells of a 24-well plate. Alternatively, single aggregates may be transferred to a new 96-well plate (suspend in 150–200 μL maturation medium containing Matrigel). By this time point, aggregates in the bacterial plate should be visible without use of a microscope.
8. Beginning on day 10 and continuing every other day (day 12, 14, 16, etc.), replace half of the medium in each well with maturation medium that does not contain Matrigel. For aggregates plated in 96-well plates, replace half of the medium every day beginning on day 9. Aggregates can be incubated for up to 22 days in 24-well plates, or 12 days in 96-well plates. This results in a total optimal culture period of 30 days (in 24-well plates) or 20 days (in 96-well plates). Aggregates can be fixed at any point for imaging as a cryosection or as a whole mount. After the prescribed durations, further culture will likely require changes in culture medium or format.

3.7 Preparation of Aggregates for Immuno-histochemistry

1. Using a P1000 tip (for collection days 1–6) or a wide-mouth P1000 tip (for collection after day 6), pipet aggregates into a 2 mL tube.
2. Aspirate the medium and add 4 % PFA (vol/vol) in PBS to the tube (*see* **Note 12**).
3. Incubate the aggregates in 4 % PFA to fix the tissue. For aggregates collected day 1–8, incubate for 20 min at RT. For day 9–30 aggregates, incubate overnight at 4 °C.
4. Remove PFA and wash aggregates at least three times with PBS. Fixed aggregates can be stored in PBS at 4 °C for several months (*see* **Note 13**).
5. Incubate the aggregates in a series of sucrose–PBS solutions of increasing sucrose concentration (i.e., 10, 20, 30 % (wt/vol) sucrose). Incubate in each concentration for 30 min while rocking. Sucrose treated aggregates can be stored at 4 ° C for up to 1 week.
6. Using a wide-mouth pipette, transfer the aggregates into a cryomold and carefully remove the sucrose solution using a P20 pipette. Gently position the aggregates in the center of the cryomold.
7. Slowly add tissue-freezing medium along the outer walls of the cryomold, taking care to make sure the aggregates do not float away from the bottom of the cryomold.
8. Place the cryomolds in a vacuum desiccator for 30 min. This will remove bubbles from the tissue-freezing medium.
9. Embedded aggregates should be placed on dry ice for 30 min, or until the tissue-freezing medium becomes opaque. Place frozen tissue blocks in a sealed container or zip-lock bag and store at −80 °C. Frozen tissue blocks can be stored for several years.
10. Using a cryostat, cryosection tissue blocks into sections of 10–15 μM in thickness.
11. Cryosections may now be analyzed using a standard immuno-histochemistry protocol (*see* **Note 14**).

3.8 Preparation of Aggregates for Whole-Mount Staining

1. Collect and fix the tissue, as described in **steps 1–4** of Section 3.7.
2. Add PBS containing 10 % (vol/vol) normal goat serum and 0.1 % (vol/vol) Triton X-100 to block the fixed aggregates. Shake overnight at RT. Note that BSA or normal horse serum can be used in place of goat serum, depending on the type of antibody being used.
3. Remove the blocking solution and apply primary antibody solution: PBS containing 3 % (vol/vol) normal goat serum,

0.1 % (vol/vol) Triton X-100, plus primary antibodies. Shake for 2–3 days at RT.

4. Remove primary antibody solution and suspend aggregates in PBS containing 0.1 % (vol/vol) Triton X-100 to wash. Shake for 1 h at RT. Repeat three times.
5. Apply secondary antibody solution: PBS containing 3 % (vol/vol) normal goat serum, 0.1 % (vol/vol) Triton X-100, plus secondary antibodies. Shake for 2–3 days at RT (*see* **Note 15**).
6. Repeat **step 4**.
7. Apply scaleA2 solution to aggregates for 4–5 days prior to imaging (*see* **Note 16**).
8. To image, transfer ~250–500 μL of scaleA2 solution containing an aggregate to an imaging chamber. The volume of solution needed will be determined by the size of the imaging chamber used.
9. Affix the coverslip slowly; note that a small amount of liquid may escape. If an air bubble should form, it will likely not interfere with imaging.
10. Flip the chamber while imaging in order to view the specimen from multiple orientations.

4 Notes

1. It is important that the LIF-2i maintenance medium is free of vitamin A. Vitamin A is capable of converting into retinoic acid, which can cause spontaneous differentiation in culture. When adapting ES cells to culture in LIF-2i medium, serial passaging can eliminate any undesirable differentiation that may arise.
2. Note that during the maturation phase of culture, penicillin–streptomycin (a component of ectodermal differentiation medium) is replaced with normocin. Streptomycin is an aminoglycoside, which can be toxic to inner ear hair cells (27). Normocin does not contain aminoglycosides. Alternative choice is Ampicillin, which is also devoid of aminoglycosides.
3. Coating the plate with gelatin prior to plating promotes adherence of cells to the surface of the plate. If ES cells do not attach to the plate, it may be due to insufficient gelatin coating. Re-plate the cells on a new plate with fresh gelatin coating.
4. If there is an issue with cell attachment or growth, the cells may not be adapted to growth in LIF-2i medium. In this case, we recommend passaging the cells several times before use.
5. Take care to wash thoroughly with PBS, as residual LIF-2i medium can affect differentiation.

6. This step is important for ensuring that the cells are completely dissociated into single cells. Take care to pipet forcefully through the cell-top strainer.
7. For example, if the cell suspension contains 1×10^6 cells, dilute 0.66 mL of cell suspension in 21.34 mL ectodermal differentiation medium.
8. Matrigel is critical for the formation of an epithelium on the surface of the aggregates. If an epithelium does not form or thicken over time, Matrigel may not have been properly incorporated into the ectodermal differentiation medium. It is essential to add Matrigel to cold medium and mix immediately for complete incorporation.
9. Non-neural ectoderm is induced via the application of BMP4. Simultaneous application of SB-431542, a TGF-β inhibitor, serves to block the formation of mesoderm.
10. FGF-2 and LDN application are necessary for induction of pre-placodal ectoderm. LDN serves to attenuate BMP signaling.
11. Wide-mouth P1000 tips are most effective in the transfer of day 8 aggregates. To prepare, use scissors to cut approximately 2 mm off the end of a tip. A whole box of tips may be prepared in this manner, and autoclaved prior to use.
12. It is important to note that PFA is toxic. Apply and remove PFA in a fume hood.
13. For long-term storage, suspend aggregates in PBS containing 0.02 % (wt/vol) sodium azide. This will suppress bacterial growth.
14. A table of antibodies for markers of placodal and otic development may be found in Koehler et al. (28).
15. Exposure to light may cause photo-bleaching of the fluorophores. To protect samples, wrap samples in foil during this and subsequent steps.
16. Note that scaleA2 medium may cause slight enlargement of the tissue.

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Differentiation of Adipocytes in Monolayer from Mouse Embryonic Stem Cells

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Abstract

Obesity and its comorbidity incidence have increased worldwide during the past 10 years. In consequence, researchers have drawn their attention to the understanding of adipocyte differentiation. Several cellular model systems have been established; however no efficient protocol could be developed so far to differentiate the pluripotent embryonic stem cells to adipocytes. In this chapter, we describe a detailed protocol that is optimized for mouse embryonic stem cells. The result of this differentiation is a homogenous adipocyte monolayer culture that can be used for several applications including developmental and pharmacological research.

Keywords: Adipocyte differentiation, Mouse embryonic stem cell, Monolayer culture, Ascorbic acid

1 Introduction

Obesity has become a public health issue worldwide due to the increasing incidence and prevalence of overweight patients and related comorbidities. In the USA 39 % of the population is obese (1). These obesity-linked diseases affect quality of life and increase mortality rate in the affected population (2, 3). Therefore, understanding adipocyte differentiation regulation has drawn the interest of researchers. Two phases of fat cell differentiation process can be distinguished: (1) determination phase, which includes the stem cell differentiation to a preadipocyte stage, and (2) terminal differentiation phase that involves the events of the differentiation from preadipocyte to adipocytes (4). There are various models for adipocyte differentiation that focus in the terminal differentiation stage (4, 5). However, the ideal model system for adipocyte differentiation would encompass both the early and the late stages. In theory, somatic stem cells and pluripotent stem cells can be used for this purpose (6). It had been shown that these cells can differentiate into all three embryonic layers (7). The principal problem of this model system is the low efficiency of the adipocyte differentiation and heterogeneity of the culture (8–10). We recently described that with the addition of ascorbic acid one can overcomes these

issues (11) and here we present a step-by-step protocol with detailed instructions and notes. The adipocytes differentiated from embryonic stem cells using this protocol can be used for various aims including transcriptomic, proteomic studies and provide a novel model system for pharmacological research.

2 Materials

1. mESC medium: Dulbecco's modified essential medium (DMEM) with GlutaMAX (Gibco, 31966-021) supplemented with 15 % of Hyclone FBS (Thermo, SH30070.03), 100× Non-Essential Amino Acid solution (Sigma, M7145), 100× Penicillin/Streptomycin (PAA, P11-010), 0.1 mM 2-mercaptoethanol (Sigma, M3148), and 1,000 U/mL of Leukemia Inhibitory Factor (Millipore, ESG1107) (*see* **Note 1**).
2. Differentiation medium: DMEM with GlutaMAX (Gibco, 31966-021) supplemented with 10 % of Hyclone FBS (Thermo, SH30070.03), 100× Non-Essential Amino Acid solution (Sigma, M7145), 100× Penicillin/Streptomycin (PAA, P11-010) (*see* **Note 1**).
3. Feeder's medium: DMEM (Sigma, D5671) supplemented with 10 % of FBS, 100× Penicillin/Streptomycin (PAA, P11-010), 100× L-glutamine (PAA, M11-004).
4. Feeder's freezing medium: 1:1 ratio of feeder's medium and DMEM (Sigma, D5671) supplemented with 20 % of cell culture-tested FBS and 10 % DMSO.
5. 0.05 % Trypsin-EDTA (Gibco, 25300-054) (*see* **Note 2**).
6. Primary mouse embryonic fibroblasts (PMEFs), mitomycin C treated: PMEFs are routinely isolated from 13.5-day-old mouse embryos (strain: C57BL/6 or CD-1). Isolated cells are cultured in 150 mm culture dishes and expanded by passaging them up to two times in a dilution 1:4. Before freezing, the cells are treated with 10 μg/mL mitomycin C (Sigma, M0503) for 3.5 h. After the mitomycin C treatment the PMEFs are washed twice with PBS and trypsinized. The cellular suspension is centrifuged for 325 × *g* and resuspended in feeder's freezing medium and aliquoted by adding 600,000 cells in each cryovial (*see* **Note 3**). PMEFs mitomycin C treated are also commercially available (Millipore, PMEF-CF).
7. Mouse embryonic stem cells: The described protocol has been optimized to E14 cells. Modified E14 cell lines are commercially available (ATCC® CRL-1821™).
8. Dimethyl-sulfoxide (DMSO) (Sigma, D5879).
9. Methanol (Analar NORMAPUR, 20847.295).

10. Absolute Ethanol (Analar NORMAPUR, 20821.296).
11. Sterile nuclease-free water (NFW).
12. All-trans retinoic acid (ATRA) (Sigma, R2625): Prepare a 1 mM stock in DMSO, keep in the dark, and store it at −20 °C.
13. L-Ascorbic acid (AsA) (Sigma, A4403) 12.5 and 25 mg/mL stock in NFW: Store it at −20 °C up to 1 month, keeping it in dark conditions (*see* **Note 4**).
14. Rosiglitazone (Selleckchem, S2505): Prepare 1 mM stock in DMSO:ethanol.
15. 3-Isobutyl-1-methylxanthine (IBMX) (Sigma, I5879) 0.5 M stock in DMSO:ethanol (*see* **Note 5**).
16. Insulin solution 10 mg/mL (Sigma, I9278): Dilute it in differentiation medium to 1 mg/mL.
17. Phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4 mM Na_2HPO_4, and 1.46 mM KH_2PO_4).
18. 3,3′,5-Triiodo-L-thyronine sodium salt (T3) (Sigma, T6397): Prepare a 3 μM stock in differentiation medium, and aliquot it to avoid freeze-thaw cycles.
19. Dexamethasone (Sigma, D4902) 0.1 mM stock in ethanol: It is very stable at −20 °C.
20. Indomethacin (Sigma, I7378) 30 mM stock in methanol: Can be stored at 4 °C up to 1 month.
21. Accutase (PAA, L11-007).
22. Sterile gelatin, 0.1 %.
23. Cell culture dishes 100 mm, 150 mm (Greiner Bio-One, 664160 and 639160, respectively) and 6-well plates (TPP, TPPA92006).
24. Cell Strainer, 40 μm (BD Bioscience, 352340).
25. Bacteriological grade dishes 150 mm (SARSTEDT, 82.1184).
26. 15 and 50 mL conical sterile tubes.
27. 10 mL pipette tips.

3 Methods

All procedures should be done under sterile conditions using aseptic techniques. Appropriated cell culture hood should be used. All centrifugations are carried out at 325 × *g* during 5 min. Standard 37 °C humidified 5 % CO_2 incubator is needed.

3.1 mES Cell Culture

1. Prepare gelatinized plates for PMEFs. Add 1 mL 0.1 % gelatin to 2 wells of a 6-well plate. Incubate at least for 30 min at 37 °C in humidified incubator (*see* **Note 6**). Thaw one cryovial of

PMEFs, and immediately remove the freezing medium containing DMSO by centrifugation. Resuspend the cell pellet in feeder's medium and plate them into the previously gelatinized wells after removing the gelatine (*see* **Note 7**).

2. 12 or 24 h after preparing the feeder's monolayer thaw 1,000,000 mES cells. To remove DMSO freezing medium transfer the content of the cryovial into a 15 mL centrifuge tube containing 4.5 mL mESC medium and centrifuge the cells. Resuspend gently the cell pellet in 4 mL fresh mESC medium and plate the cells in the 2 wells containing mitomycin C-treated PMEFs (*see* **Note 8**). Change medium daily using mESC medium.
3. Under these conditions the cells should be ready to use after 3 days in culture. The mES cells should reach 70–80 % confluency (Fig. 1a).

3.2 mES Cell Feeder Depletion

1. As a first step of differentiation split the culture in 1:5 ratio in feeder-free condition. First, wash once the cells with PBS, add trypsin, and incubate for 3–5 min in the incubator. Add mESC medium to inactivate the trypsin and dissociate the stem cell clumps until single-cell suspension is achieved. Then split in 1:5 ratio (*see* **Note 9**). Change mESC medium daily. The feeder-free stem cells change their morphology (Fig. 1b) but they keep their high capacity of cell proliferation.
2. After 2 days of culture the feeder-free mES cells can be passaged. Repeat the feeder-free passage two times more. As the last two feeder-free passages can be done in 1:10 or 1:15 dilution, here cells can be expanded to the needed amount (*see* **Note 10**).

3.3 Embryonic Body (EB) Formation in "Hanging Drop" Method

1. The feeder-free stem cells are trypsinized. The cellular suspension should be counted and diluted for EB formation. Briefly, before adding trypsin to the cell culture wash once with PBS. The trypsin is inactivated by 4 mL differentiation medium per 1 well of 6-well plate (*see* **Note 11**). Count the total cell number and prepare a cell suspension by diluting the cells in differentiation medium to 1×10^5 cells/mL. Prepare the bottom of 150 mm bacteriological Petri dish by adding 15 mL PBS and 100 μL differentiation medium. The latter step will decrease the surface tension of the liquid. Finally, place 20 μL drops of the 1×10^5 cells/mL suspension onto the inner surface of the lids of bacteriological grade dishes with a multichannel pipette. This step is referred as "Adipocyte differentiation Day 0" (Fig. 1c). Invert the lid over the bottom of the bacteriological Petri dish and place it very carefully in the incubator.

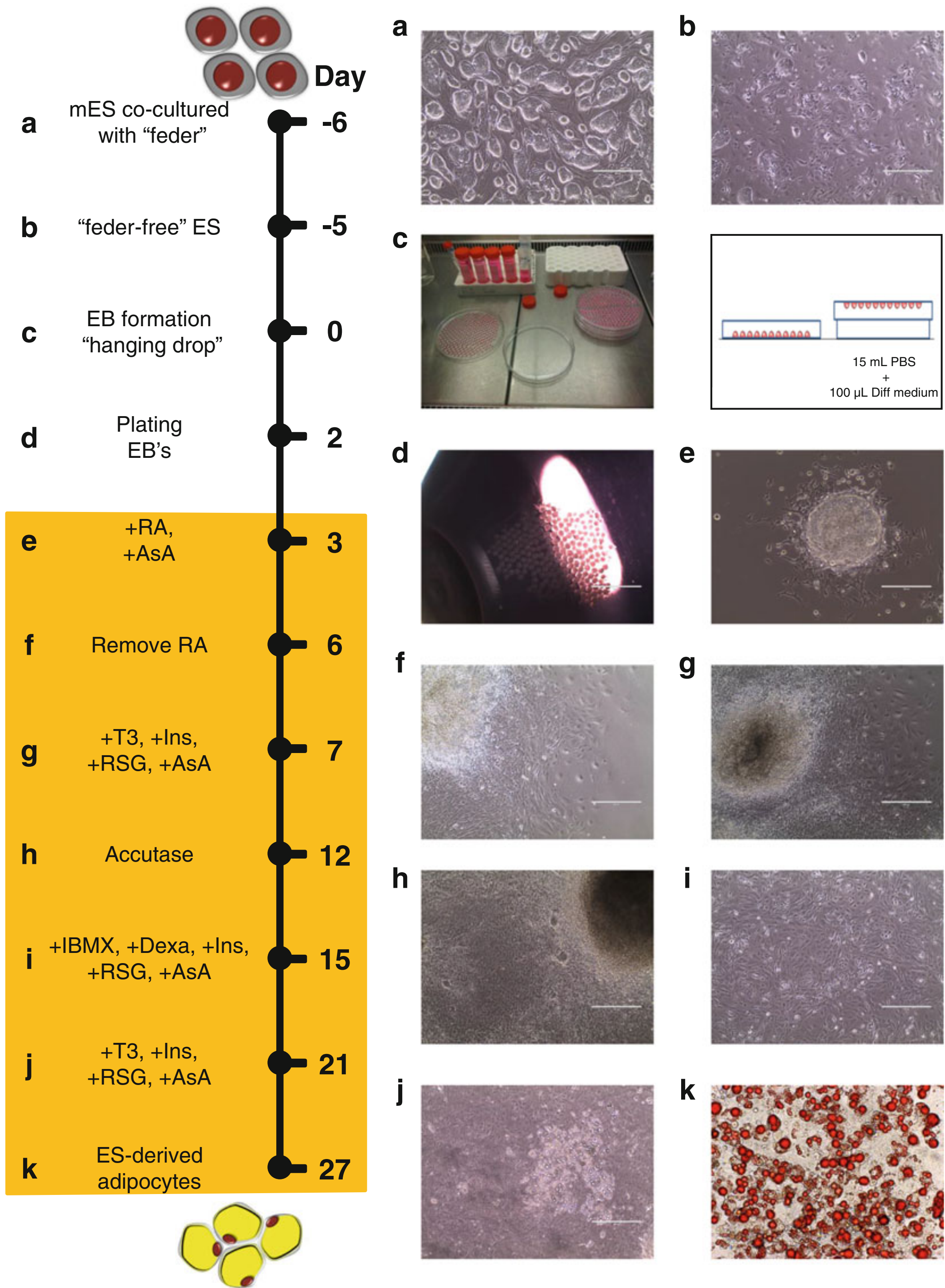

Fig. 1 Stepwise adipocyte differentiation protocol from mouse embryonic stem cells. (**a**–**k**) is a representative picture of the steps mentioned in the text

2. After 2 days the EBs are collected. Slowly remove the lid of the bacteriological plates, invert it, and wash the lids with 10 mL PBS to collect the EBs into a 50 mL conical sterile tube. Remove the medium by allowing EBs to sediment for 10–20 min at room temperature (day 2, Fig. 1d). Aspirate the PBS and resuspend the EBs in differentiation medium. The EBs obtained from one lid can be plated in 1 well of gelatin-precoated 6-well plate in 1 mL differentiation medium (*see* **Note 12**). Place back in the incubator.

3.4 Induction of Adipocyte Differentiation of Plated EBs

1. One day after plating the EBs (at day 3), change differentiation medium containing only 1 μM ATRA and 12.5 μg/mL AsA in final concentration and change medium every day (Fig. 1e) (*see* **Note 13**).
2. Three days after starting the ATRA treatment (day 6, Fig. 1f) remove ATRA from the medium. Table 1 contains a summary of medium and supplements used along the differentiation.
3. Next day (day 7, Fig. 1g), change the differentiation medium by adding the freshly prepared hormonal cocktail: AsA (12.5 μg/mL), T3 (3 nM), insulin (0.5 μg/mL), and

Table 1
Summary of medium and adipogenic cocktails in mES adipocyte differentiation

Days of differentiation	Medium	Supplemented with
Until day 0	mESC medium	LIF
Day 0 to day 3	Differentiation medium	
Day 3 to day 6	Differentiation medium	ATRA 1 μM AsA 12.5 μg/mL
Day 6 to day 7	Differentiation medium	AsA 12.5 μg/mL
Day 7 to day 15	Differentiation medium	Rosiglitazone 0.5 μM[a] Insulin 0.5 μg/mL T3 3 nM AsA 12.5 μg/mL
Day 15 to day 21	Differentiation medium	IBMX 0.5 μM Dexamethasone 0.1 μM Insulin 20 μg/mL Rosiglitazone 0.5 μM[a] Indomethacin 0.06 mM AsA 25 μg/mL
Day 21 to day 27	Differentiation medium	Insulin 20 μg/mL Rosiglitazone 0.5 μM[a] AsA 25 μg/mL T3 3 nM

Leukemia inhibitory factor (LIF), All-trans retinoic acid (ATRA), Ascorbic acid (AsA)
[a]The addition of rosiglitazone is optional; *see* **Note 14** for details

rosiglitazone (0.5 μM). Change this medium daily; prepare every day freshly from stock solutions. The addition of rosiglitazone is optional (*see* **Note 14**).

4. At day 12 of adipocyte differentiation (Fig. 1h) disperse the cells by 1 mL accutase. Before the enzymatic digestion wash the culture with PBS once. Incubate the cells with accutase for 20–25 min in the incubator. Add 2 mL differentiation medium, resuspend thoroughly, and place the cellular suspension in a 50 mL sterile conical tube. Finally wash each well with 2 mL differentiation medium and collect it on the same tube (*see* **Note 15**). Plate the cells in differentiation medium supplemented with AsA (12.5 μg/mL), rosiglitazone (0.5 μM), insulin (0.5 μg/mL), and T3 (3 nM) (*see* **Note 16**).
5. Do not change the medium between days 12 and 15 of the differentiation.
6. After day 15 (Fig. 1i) change medium every 3 days supplemented with AsA (25 μg/mL), dexamethasone (0.1 μM), insulin (20 μg/mL), rosiglitazone (0.5 μM), indomethacin (0.06 mM), and IBMX (0.5 mM). The addition of rosiglitazone is optional (*see* **Note 14**).
7. At day 21 of adipocyte differentiation (Fig. 1j) and day 24 change differentiation medium containing AsA (25 μg/mL), insulin (20 μg/mL), rosiglitazone (0.5 μM), and T3 (3 nM). The addition of rosiglitazone is optional (*see* **Note 14**).
8. Assess the cultures for the presence of lipid droplet containing adipocytes at day 27 (Fig. 1k) (*see* **Note 17**).

4 Notes

1. The quality of the medium and serum used during the stem cell culture is a crucial factor for the differentiation process. We recommend using the mentioned medium and serum for culturing the stem cells and for the differentiation. If another medium or serum would be used it is very important not only to assess the morphology of the stem cells during expansion but also the ability to form EBs and differentiate. LIF should be added to the medium freshly or the mESC medium containing LIF should be used preferably within 3 days.
2. Either for expansion or EB formation, after chemical disruption of the stem cell culture the cells should be in a single-cell suspension. In our experience not all the brands of trypsin achieve this and we recommend the 0.05 % trypsin mentioned in Section 2.
3. The freezing medium for the mitomycin-treated PMEFs is important for their survival. Also the freezed mitomycin-treated PMEFs should be shortly collocated in liquid nitrogen (within 1

or 2 days after freezing) for longer term storage. PMEFs should be tested for mycoplasma in case of each new preparation.

4. Ascorbic acid stock should not be used after 1 month of storage. It is a light-sensitive compound. It is recommended to prepare a new stock for each differentiation process. Similar results were obtained using sodium L-ascorbate (A4034) and L-ascorbic acid 2-phosphate sesquimagnesium salt (Sigma, A8960).
5. DMSO can inhibit the adipocyte differentiation; therefore concentrated stocks are recommended. IBMX 0.5 M stock can be difficult to resuspend; it is advisable to heat it in the 37 °C water bath until the solution is completely homogeneous and store it in aliquots to avoid repeated freeze-thaw cycles.
6. Gelatin 0.1 % incubation times less than 30 min before plating PMEFs can lead to suboptimal attaching ratio of mitomycin-treated PMEFs.
7. In our experience 600,000 PMEFs mitomycin C treated can perfectly be used in 2 wells of a 6-well plate or in one T-25 flask. The cells can be used from 12 h up to 1 week after thawed.
8. Thawing 1,000,000 mES cells can be done in 2 wells of 6-well plate or in one T-25 flask.
9. The cell culture should be split 1:5 or 1:7 ratio for the first passage without feeders. mES cells are sensitive to feeder depletion; thus higher dilutions could result in stem cell spontaneous differentiation.
10. Other mES cell strains might be sensitive to feeder-free conditions as for example induced pluripotent stem cells. In such case feeder-free passages are not recommended. These stem cells should be feeder depleted the day 0 of the differentiation. Briefly, after trypsinizing the stem cell culture, the cells are incubated in a gelatinized wells or flask for 20–30 min. Pipette the cell suspension to a 15 mL centrifuge tube and count the cell number. After this step follow the EB formation step.
11. Single-cell suspension is needed for a reproducible and adequate EB formation.
12. EBs are very sensitive to mechanical manipulation. Use 10 mL pipette tips when collecting and plating the EBs. Avoid pipetting EBs several times.
13. One-day plated EBs could detach easily; therefore, remove the medium with pipette instead of water pump/vacuum suction at least in the first 3 days.
14. Addition of rosiglitazone is optional; it does not significantly increase the adipocyte differentiation in stem cells, but can produce increased differentiation of Ucp-1+ adipocytes in the terminally differentiated cultures (11).

15. If the extracellular matrix cannot be disrupted, use a 40 μm mesh to remove the clumps.
16. This protocol offers the advantage that can be used for several applications. The cells can be repleted in different dishes and well-plate formats: 100,000 cells per well of a 6-well plate, 50,000 cells per well of a 24-well plate, 1,000,000 cells per 100 mm dish, and 1,800,000 cells per 150 mm cell culture dish (11).
17. The adipocyte cultures, due to their high content of adipocytes, and the monolayer culture can be evaluated using standard techniques including transcriptome, epigenetic, and immuno-staining analysis.

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Pancreatic Differentiation from Murine Embryonic Stem Cells

Daisuke Sakano, Nobuaki Shiraki, and Shoen Kume

Abstract

Pluripotent stem cells are considered as a cell source for replacement therapies for pancreatic beta cells and other organs.

We identified tetrabenazine (TBZ), vesicular monoamine transporter 2 (VMAT2) inhibitor as a promoter of late-stage differentiation of *Pdx1*-positive pancreatic progenitor cells into *Ngn3*-positive endocrine progenitor cells. A cell-permeable cAMP analog, dBu-cAMP promotes beta cell maturation in late stage of differentiation. The induced beta cells can secrete insulin in a glucose-dependent manner.

Our protocol consists of a three -step differentiation process. ES cell recapitulate embryonic developmental processes in vitro. Therefore, the ES cell differentiation system is a useful model for the understanding of molecular mechanism of beta-cell differentiation and are useful for application for future regenerative medicine.

Keywords: Pancreatic differentiation, VMAT2 inhibitor, GSIS

1 Introduction

The differentiation into pancreatic β cells is through the generation of definitive endoderm. Differentiation into definitive endoderm is promoted by Nodal and Activin, both are members of the TGFβ superfamily (1, 2). In in vitro differentiation of human ES/iPS cells, treatment with activin A at the presence of a GSK3β inhibitor efficiently induced endodermal differentiation (3, 4). Then, definitive endoderm cells further differentiate into Pdx1 (Pancreatic and duodenal homeobox 1)-positive pancreatic progenitor cells, which give rise to pancreatic duct, exocrine and endocrine cells. Cyclopamine, a Shh inhibitor, retinoic acid (RA), and addition of Noggin are reported to be important factors for the induction of pancreatic progenitor cells from human ES/iPS cells (5–8).

Small compounds are utilized instead of adding growth factors themselves. For example, LDN-193189 is used instead of adding Noggin. SANT-1, a Smo and Sonic Hedgehog signaling pathway inhibitor, is used to substitute cyclopamine (8).

Plasma membrane-permeable low molecular weight compounds are cost-effective and thus are more practical compared to growth factors. Large-scale screenings of chemical libraries have identified inducer of the definitive endoderm (IDE1) and IDE2 as positive regulators for differentiating ES cells to the definitive endoderm (9). Similarly, Indolactam V, a protein kinase C activator, is identified to promote the differentiation from definitive endoderm into the pancreatic progenitor (10). Addition of forskolin (protein kinase C inhibitor), dexamethasone (agonists of glucocorticoids), TGF-β signaling inhibitors promote the differentiation from pancreatic progenitor cells to β cells, although the induced β cell exhibit immature insulin secretion ability (11).

Through a large-scale screening, we identified two chemical compounds that promote differentiation into beta cells that can secrete insulin in a glucose-dependent manner. In our screen, TBZ, an inhibitor of vesicular monoamine transporter 2 (VMAT2) was identified to promote efficient differentiation from pancreatic progenitor cells to Ngn3-positive endocrine progenitor cells (Fig. 1). VMAT2 controls uptake of monoamines, namely dopamine histamine and serotonin, into intracellular vesicles. VMAT2-monoamine signaling negatively controls the differentiation of β cell. A cell permeable cAMP analog, dBu-cAMP, enhances beta-cell maturation into cells capable of glucose-sensitive insulin secretion. Application of VMAT2 and dBu-cAMP simultaneously showed synergistic effects, thereby can contribute to the regenerative medicine (12).

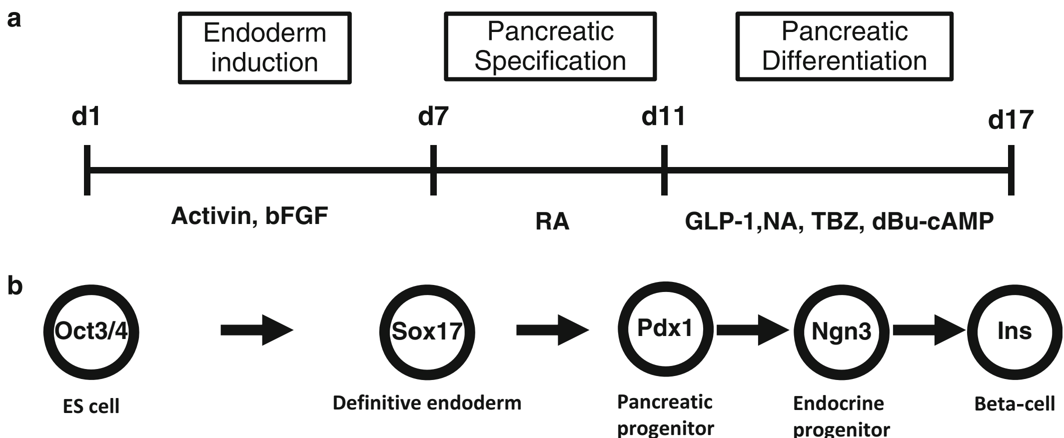

Fig. 1 Scheme for pancreatic differentiation procedure. (**a**) A schematic drawing of the differentiation schedules. Time windows for sequential application of specific growth factor. *bFGF* basic fibroblast growth factor, *RA* retinoic acid, *NA* nicotinamide, *TBZ* tetrabenazine, *dBu-cAMP* dibutyryl-cAMP. (**b**) The corresponding developmental stages of differentiation and stage-specific marker genes

2 Materials

2.1 Murine ES Cell Differentiation

1. Culture Plate(96-well-plate, Corning Coaster UltraWeb Synthetic Polyamine Surface,3873XX1). The UltraWeb is composed of randomly orientated electro spun polyamine fiber. The fiber size is between 200 and 400 nm with average diameter size of 280 nm. The pore size of the fiber is approximately 700 nm.
2. PBS (*see* **Note 1**).
3. 0.25 % Trypsin-EDTA (Invitrogen, 25200-072).
4. ES cell plating medium.

DMEM (Invitrogen, 11995-075, high Glucose)	500 mL
Penicillin/ Streptomycin	
(P/S) (Nacalai tesque, 26252-94)	5 mL
L-Glutamine (Nacalai tesque, 16948-04)	5 mL
MEM nonessential amino acids solution	
(NEAA) (Invitrogen, 11140-050)	5 mL
0.1 M 2-mercaptoethanol (*see* **Note 3**)	500 μL
Fetal bovine serum (Hyclone)	50 mL

5. Mouse endoderm differentiation basal medium (store at 4 °C).

DMEM (Invitrogen, 11995-075, high Glucose)	500 mL
AlbuMAX II (Invitrogen, 11021-029) (*see* **Note 2**)	6 mL
Insulin-Transferrin-Selenium-G (Invitrogen, 41400-045)	5 mL
Penicillin/ Streptomycin	
(P/S) (Nacalai tesque, 26252-94)	5 mL
L-Glutamine (Nacalai tesque, 16948-04)	5 mL
NEAA (Invitrogen, 11140-050)	5 mL
0.1 M 2-mercaptoethanol (*see* **Note 3**)	500 μL

6. Supplements for mouse endoderm differentiation medium. Activin (R&D, 338-AC): Stock solution at 10 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 mouse endoderm differentiation basal medium at a final concentration of 10 ng/mL.

   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 mouse endoderm differentiation basal medium at a final concentration of 5 ng/mL.

7. Mouse pancreatic specification basal medium (store at 4 °C).

RPMI (Invitrogen, 11875-093)	500 mL
B27 supplement (Invitrogen, 17504-044)	10 mL
P/S (Nacalai tesque, 26252-94)	5 mL
L-Glutamine (Nacalai tesque, 16948-04)	5 mL
NEAA (Invitrogen, 11140-050)	5 mL
0.1 M 2-mercaptoethanol (*see* **Note 3**)	500 μL

8. Supplements for mouse pancreatic specification medium. Stemolecule™ All-Trans Retinoic Acid (ATRA; Stemgent, #130-095-571): Stock solution at 10 mM in DMSO (Sigma, D2650). Aliquot into 100 μL and store at −80 °C. Once thawed, keep at 4 °C with protection from light. Add to mouse pancreatic specification basal medium at a final concentration of 10^{-6} M.

   Human recombinant fibroblast growth factor-10 (FGF10, Peprotech, #100-26-2): Stock solution at 50 μg/mL in 0.1 % (w/v) BSA/ PBS. Aliquot into 50 μL and store at −80 °C. Once thawed, keep at 4 °C. Add to mouse pancreatic specification basal medium at a final concentration of 50 ng/mL.

   3-Keto-N-(aminoethyl-aminocaproyl-dihydrocinnamoyl) cyclopamine (KAAD-cyclopamine, stemgent, #04-0028): Stock solution at 25 μM in DMSO (Sigma, D2650). Aliquot into 50 μL and store at -80 °C. Once thawed, keep at 4 °C with protection from light. Add to mouse pancreatic specification basal medium at a final concentration of 0.25 μM.

9. Mouse pancreatic differentiation basal medium (store at 4 °C).

DMEM (Invitrogen, 11885-084, low Glucose)	500 mL
AlbuMAX II (Invitrogen, 11021-029) (*see* **Note 2**)	6 mL
Insulin-Transferrin-Selenium-G (Invitrogen, 41400-045)	5 mL
Penicillin/ Streptomycin	
(P/S) (Nacalai tesque, 26252-94)	5 mL
L-Glutamine (Nacalai tesque, 16948-04)	5 mL
MEM nonessential amino acids solution	
(NEAA) (Invitrogen, 11140-050)	5 mL
0.1 M 2-mercaptoethanol (*see* **Note 3**)	500 μL

10. Supplements for mouse pancreatic differentiation medium.

Glucagon-like peptide 1 amide fragment 7-36 (GLP-1, Sigma-Aldrich, #8147-5MG) : Stock solution at 100 μM in ultrapure water (*see* **Note 4**). Aliquot into 50 μL and store at −80 °C. Once thawed, keep at 4 °C with protection from light. Add to mouse pancreatic specification basal medium at a final concentration of 10 nM.

Nicotinamide (NA, Sigma-Aldrich, #N0636-100G) : Stock solution at 1 M in ultrapure water (*see* **Note 5**). Aliquot into 5 mL and store at −80 °C. Once thawed, keep at 4 °C with protection from light. Add to mouse pancreatic specification basal medium at a final concentration of 10 mM.

Tetrabenazine (TBZ, TOCRIS, # *2175*) Stock solution at 10 mM in DMSO (Sigma, D2650). Aliquot into 50 μL and store at −80 °C. Once thawed, keep at 4 °C with protection from light. Add to mouse pancreatic specification basal medium at a final concentration of 1.25 μM.

Dibutyryl-cAMP (dBu-cAMP, BIOMOL International, # CN125): Stock solution at 10 mM in DMSO (Sigma, D2650). Aliquot into 50 μL and store at −80 °C. Once thawed, keep at 4 °C with protection from light. Add to mouse pancreatic specification basal medium at a final concentration of 1.25 μM.

3 Methods

Carry out all procedures in clean bench and keep the cells in CO_2 incubator with 37 °C, 90 % humidity, and 5 % CO_2.

All medium and solution should be warmed up at room temperature. DO NOT use cold medium.

Take 200 μL of every differentiation medium for one well of 96-well nanofber plates.

3.1 Murine ES Cell: Plating

1. Remove ES maintenance medium.
2. Wash with 5 mL PBS for 60 mm dish
3. Add 1 mL 0.25 % trypsin-EDTA solution to 60mmφ dish. Let stand for 5 min at 37 °C and confirm under microscope for detachment of cells.
4. Disperse the cells into a single-cell suspension by pipetting with a P1000 pipet.
5. Add 4 mL mouse ES plating medium and collect the cells by centrifugation at 180 *g* for 5 min.
6. Resuspend the pellet with mouse ES plating medium, cell count, and adjust to a final cell density of 2.5×10^4 cells/mL

7. Plate 200 μL ES cell suspension into each well of 96-well synthetic nanofiber plate.
8. Incubate at 37 °C under 5 % CO_2 overnight.

3.2 Murine ES Cell: Differentiation

1. Change medium by replacing with fresh mouse endoderm differentiation medium supplemented with both activin and bFGF on day 1, 3, and 5 (*see* **Note 6**).
2. Change medium by replacing with fresh mouse pancreatic specification medium supplemented with ATRA, FGF10, and KAAD-cyclopamine on day 7 and 9.
3. Change medium by replacing with fresh mouse pancreatic differentiation medium supplemented with TBZ, dBu-cAMP, GLP-1, and NA on day 11, 13, and 15. Change medium every 2 days to extend culture period.

4 Notes

1. Dissolve three tablets PBS (SIGMA, #P4417-100TAB) in 600 mL ultrapure water, autoclave, and store at room temperature.
2. Dissolve 25 g AlbuMAX II in 125 mL ultrapure water with stirring. Sterilize it with filtration (Millipore, SCGPS05RE). Aliquot into 2 mL and store at −20 °C.
3. Dilute 2-mercaptoethanol (Sigma,M7522) to 0.1 M with PBS (i.e., 2-mercaptoethanol 100 μL/ PBS 14.1 mL). Store at 4 °C and use within 1 month.
4. Dissolve 5 mg Glucagon-like peptide 1 amide fragment 7–36 with 1516 μL ultrapure water. Sterilize with filtration (Millipore, SCGPS05RE). Aliquot into 50 μL and store at −80 °C.
5. Dissolve 12.2 *g* Nicotinamide with 100 mL ultrapure water. Sterilize with filtration (Millipore, SCGPS05RE). Aliquot into 5 mL and store at −80 °C.
6. To change medium, as cells attached on the nanofiber surface weakly, dispense the fresh medium gently not to blow them up.

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Osteogenic Differentiation from Embryonic Stem Cells

Yanhong Yu, Carlos Pilquil, and Michal Opas

Abstract

Embryonic stem (ES) cells have been widely studied due to their pluripotency and their potential of self-renewal. Murine ES cells are useful in investigating the molecular pathways underlying their differentiation to various mature cell types in the body. This chapter describes the maintenance of murine ES cells in culture and a routine ES cell osteogenic differentiation protocol utilized in our laboratory.

Keywords: Murine embryonic stem cells, Routine culture, Hanging drop, Embryoid bodies, Osteogenesis

1 Introduction

Since their first isolation by two independent laboratories (1) two decades ago, murine embryonic stem (ES) cells have been extensively studied. This work in turn has revolutionized the fields of regenerative medicine, genetics, and developmental biology. This tremendous interest in the field of ES cells is due to their potential to differentiate to all types of mature cells in the body and their ability of unlimited self-renewal in vitro. ES cells are isolated from the inner cell mass of blastocysts in the pre-implanted stage. Under certain culture conditions, they can differentiate and give rise to the three germ layers: the endoderm, mesoderm, and ectoderm. ES cells are commonly propagated on a layer of mitomycin-treated, and thus division-incompetent mouse fibroblasts, which have been shown to be critical in maintaining the undifferentiated state of ES cells (2). A soluble factor, leukemia inhibitory factor (LIF), is also added to prevent the spontaneous in vitro differentiation of ES cells. The withdrawal of LIF from culture and growth in suspension causes differentiation and embryoid body (EB) formation (3).

EBs are a unique in vitro model which allows for the study of early embryonic development such as the generation of the three germ layers. They are three-dimensional aggregates of differentiating stem cells. There are many different ways one can generate EBs: growth in suspension culture using low-adherence vessels or bacteriological dishes, bioreactor/spinner flask techniques, culture using methylcellulose media, and hanging drops (4). The hanging drop

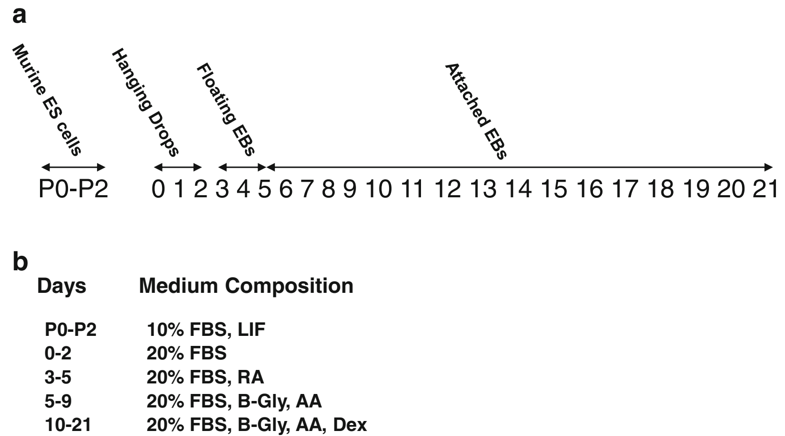

Fig. 1 ES cell maintenance and osteogenic differentiation. (**a**) Murine ES cells are maintained on mouse embryonic fibroblast feeder cells for two passages. At passage 2, ES cells form EBs via the hanging drop method. From days 3 to 5, they grow in suspension. They are attached on gelatinized tissue culture dishes on day 6 and grow for the remainder of the 21 days. (**b**) Different medium composition is required for different stages of ES cell maintenance and osteogenic differentiation. (Abbreviations: *ES* embryonic stem, *EB* embryoid body, *FBS* fetal bovine serum, *LIF* leukemia inhibitory factor, *RA* retinoic acid, *B-Gly* β-glycerophosphate; *AA* ascorbic acid, *Dex* dexamethasone)

method is one of the more widely utilized protocols that have successfully generated various mature cell types (cardiomyocytes, neuronal cells, adipocytes, osteoblasts, etc.) (5–8). In brief, 20–30 μL of solution containing ES cells is pipetted on Petri dish lids. These lids are then inverted and placed over the Petri dish. Due to gravity, the ES cells in solution then collect at the bottom of the drops and aggregate to form EBs.

In this chapter, we describe routine maintenance and propagation of murine ES cells in vitro, and illustrate in detail the hanging drop differentiation method. As an example of differentiation, we demonstrate the osteogenic induction and differentiation of ES cells (Fig. 1).

2 Materials

2.1 Tissue Culture

2.1.1 Reagents and Equipments

1. 10 cm Tissue culture dish, 100 × 20 mm, polystyrene (Falcon, cat. no. 353003).
2. 6 cm Tissue culture dish, 60 × 15 mm, polystyrene (Falcon, cat. no. 353002).
3. Hanging drop petri dish, 150 × 15 mm, polystyrene (VWR, cat. no. 25384-326).
4. Bacteriological plates (VWR, cat. no. 25389-328).

5. 15 mL Centrifuge tube, polypropylene (FroggaBio, cat. no. TB15-25).
6. Phosphate-buffered saline (PBS): 10× stock, 76.5 g sodium chloride (NaCl) (Bioshop, cat. no. SOD002.205), 7.25 g sodium phosphate dibasic anhydrous (Na_2HPO_4) (Sigma-Aldrich, cat. no. RES20908), 2.12 g potassium phosphate dibasic anhydrous (KH_2PO_4) (Sigma-Aldrich, cat. no. P0662), 1 L distilled water (H_2O). For 1× PBS, dilute stock 1:10 with distilled H_2O. Autoclave and store at room temperature.
7. 0.1 % gelatin: 0.5 g gelatin (Anachemia, cat. no. AC-4622) in 500 mL PBS. Autoclave and store at room temperature.
8. Trypsin/EDTA: 0.25 % trypsin 2.21 mM EDTA tetrasodium (Multicell, Wisent, cat. no. 325-043-CL). Store at −20 °C.
9. Freezing vial: CryoELITE Cryogenic vials (Wheaton, cat. no. W985862).
10. Mytomycin C (Sigma-Aldrich, cat. no. M4287-2mg), stock 1 mg/mL, store at −20 °C.
11. Leukemia inhibitory factor (LIF): stock 10 μg/mL. Store at −20 °C.
12. 0.4 % Trypan blue solution (Life technologies, cat. no. 15250061).
13. Quick-Read Precision Cell, with 18 counting circles, 10 tests/slide (Globe Scientific Inc., cat. no. 010G18).
14. 8-Channel pipette (Rainin, 20–200 μL, cat. no. L8-200XLS+).
15. Retinoic acid (Sigma-Aldrich, cat. no. R625): 10 mM stock, store at −20 °C.
16. β-Glycerophosphate (Sigma-Aldrich, cat. no. G9422): stock 2 M, store at −20 °C.
17. Ascorbic acid (Sigma-Aldrich, cat. no. A4403): stock 5 mg/mL, store at −20 °C.
18. Dexamethasone (Sigma-Aldrich, cat. no. D4902): stock 0.1 mM, store at −20 °C.

2.1.2 Medium Preparation

1. 10 % FBS medium: 500 mL DMEM with sodium pyruvate and L-glutamine (Multicell, Wisent, cat. no. 319-005-ES), 50 mL Fetal Bovine Serum (FBS) (Multicell, Winsent, cat. no. 080150), 5 mL MEM nonessential amino acids 100× (Gibco, Life Technologies, cat. no. 11140-050), 500 μL diluted 2-mercaptoethanol, 35 μL 2-mercaptoethanol (Bioshop, cat. no. CAS60-24-2) in 5 mL autoclaved distilled H_2O.
2. Freezing medium: 8 mL 10 % FBS medium, 1 mL FBS, 1 mL dimethyl sulfoxide (DMSO) (Sigma-Aldrich, cat. no. D2650).
3. 17 % FBS medium: preparation same as 10 % FBS medium except for the addition of 100 mL FBS.

2.2 Von Kossa Assay

1. Formaldehyde solution, 36.5–38 % in water (Sigma-Aldrich, cat. no. F8775): store at room temperature.
2. Silver nitrate (Bioshop, cat. no. SIL222).
3. Na_2HPO_4 (Sigma-Aldrich, cat. no. RES20908).
4. Sodium phosphate monobasic monohydrate ($Na_2HPO_4 \cdot H_2O$) (Sigma-Aldrich, cat. no. S9638).

2.3 RNA Isolation, Reverse Transcription, Real-Time Polymerase Chain Reaction (PCR)

1. RNeasy Plus Mini Kit (Qiagen, cat. no. 74134).
2. 1.5 mL Eppendorf tube (Axygen, cat. no. PMI11006v2).
3. Oligo$(dt)_{18}$ Primer 0.5 μg/μL: (Thermo Scientific, cat. no. SO132). Store at −20 °C.
4. dNTP mix, 10 mM (Invitrogen. cat. no. 18427-013). Store at −30 °C.
5. UltraPure Distilled water, DNase, RNase, free (Invitrogen, cat. no. 10977-015).
6. 5× First strand buffer (Invitrogen. cat. no. P/N y02321). Store at −20 °C.
7. 0.1 M DTT (Invitrogen. cat. no. P/N y00147). Store at −20 °C.
8. RNaseOUT Recombinant Ribonuclease Inhibitor, 40 U/μL (Invitrogen. cat. no. P/N 100000840). Store at −20 °C.
9. SuperScript II Reverse Transcriptase, 200 U/μL (Invitrogen. cat. no 18064-014). Store at −20 °C.
10. Power SYBR Green PCR Master Mix (Applied Biosystems, cat. no. 4367659). For short-term storage, keep in 2–8 °C. For long-term storage, store at −20 °C.
11. 384 well plate, MicroAmp Optical Reaction Plate with Barcode (Applied Biosystems by Life Technologies, cat. no. 4309849).
12. Sealing tape, optically clear (Sarstedt, cat. no. 95.1994).
13. Osteogenic markers primers (Invitrogen): Osterix (OSX): forward primer 5′-GCAACTGGCTAGGTGGTGGTC-3′ and reverse primer 5′-GCAAAGTCAGATGGGTAAGTAGGC-3′. Collagen 1 (Col 1): forward primer 5′-GAACGGTCCACGATTGCATG-3′ and reverse primer 5′-GGCATGTTGCTAGGCACGAAG-3′. Stock 250 nM. Store at −20 °C.
14. CFX384 TouchTM Detection System (Biorad, cat. no. 185-5485).

3 Methods

3.1 Cell Culture

3.1.1 Thawing of Mouse Embryonic Fibroblasts (Feeders) Cells

1. Warm cells quickly in 37 °C incubator or water bath.
2. Add 2 mL of 10 % FBS medium in a 15 mL Falcon tube, and add the thawed cell solution in the same tube.
3. Centrifuge at 700 × *g* for 3 min.
4. In the meanwhile, add 2 mL of 0.1 % gelatin in a 6 cm tissue culture dish, cover the bottom of the dish evenly with gelatin, and aspirate gelatin. Set dish aside for later use (*see* **Note 1**).
5. Aspirate the supernatant and add fresh 2 mL 10 % FBS medium to resuspend the cell pellet in the tube (*see* **Note 2**).
6. Transfer the resuspended cell solution to the gelatinized dish, and add 2 mL 10 % FBS medium to the dish. Move dish around gently for even distribution of cells in the dish.
7. Change medium every other day. Add 4 mL of fresh 10 % FBS medium per 6 cm dish.

3.1.2 Preparation of Feeders for ES cells and Plating ES Cells

1. When feeders are at 65 % confluency, treat cells with 10 μL/mL medium of mytomycin C for 2 h to inactivate their ability to undergo mitosis.
2. After 2 h, aspirate mitomycin C, rinse cells twice with fresh 10 % FBS medium. Add 4 mL of fresh 10 % FBS medium.
3. Quickly thaw ES cells using steps 1–3 from Section 3.1.1.
4. Aspirate the supernatant and add 1 mL 10 % FBS medium to resuspend the ES cells pellet. Transfer the cell solution to the dish containing the mitomycin C-treated feeders.
5. To maintain pluripotency, add 1 μL leukemia inhibitory factor (LIF) for 1 mL medium.
6. Change medium every other day.

3.1.3 Maintenance of ES cells

1. When ES cells are at about 65 % confluency (approximately 2 days after last passage), aspirate medium, and add 1 mL trypsin/EDTA per 6 cm tissue culture dish. Incubate for 3 min. Pipette up and down a few times to break up cell clumps into single cell suspension.
2. Add 1 mL fresh 10 % FBS medium to a 15 mL Falcon tube, transfer the cell solution with trypsin into this tube.
3. Centrifuge at 700 × *g* for 3 min.
4. In the meanwhile, add 2 mL of 0.1 % gelatin in a 6 cm tissue culture dish, cover the bottom of the dish evenly with gelatin, and aspirate gelatin. Set dish aside for later use.

5. Carefully aspirate the supernatant from the 15 mL Falcon tube. Resuspend ES cell pellet in 4 mL 10 % FBS medium. Transfer this cell solution to the gelatinized dish. Add 4 μL LIF into the dish, and gently move the dish for even distribution of cells and LIF.

3.1.4 Freezing of Cells

This protocol is applicable to both feeders and ES cells.

1. Repeat steps 1–3 in Section 3.1.3. For a 10 cm tissue culture dish, use 3 mL trypsin/EDTA and add 3 mL 10 % FBS medium into the 15 mL Falcon tube.
2. Aspirate the supernatant from the 15 mL Falcon tube, Add 1 mL freezing medium, resuspend cell pellet, and transfer cells to freezing vial.
3. Store the cells at −80 °C for a day, and then keep them in liquid nitrogen for long term.

3.1.5 Preparing of EBs for Osteogenic Differentiation (Days 0–2)

1. Add 7 mL autoclaved PBS into the hanging drop petri dishes. PBS is added to prevent drying out of the hanging drops.
2. When passage 2 ES cells are at 65 % confluency, prepare for making EBs by steps 1–3 in Section 3.1.3.
3. Carefully aspirate the supernatant from the 15 mL Falcon tube. Resuspend cell pellet in 6 mL 17 % FBS medium. Gently pipette up and down to mix. Add 50 μL of cell solution to an equal volume 0.4 % trypan blue. Pipette to mix well. Let stand for 1 min.
4. Transfer 50 μL of the solution containing ES cells and trypan blue to a well on a Quick-Read Precision Cell. Count number of cells within a group of nine circles. Count cells in the another group of nine circles. Take the average of the 2 numbers, multiply by 2 to account for dilution with trypan blue, and multiply by 10^4. This is the number of cells present in 1 mL original cell solution. Repeat with another well on the same Precision Cell slide. Take the average of the number of cells calculated.
5. For osteogenic differentiation, our laboratory discovered that the optimal initial seeding number of cells per EB is approximately 250 cells. Each hanging drop dish requires approximately 3.6 mL cell solution (144 drops per dish × 25 μL medium per drop). Each dish will require 36,000 cells per dish (144 drops per dish × 250 cells per drop). Add appropriate number of cells into the medium for the desired number of hanging drop dishes. Pipette gently to mix.
6. Use an 8-channel pipette to dispense 25 μL drops of cell solution onto the lids of the Petri dishes. Carefully invert the lid and place it over the bottom of the dish. Transfer to incubator.

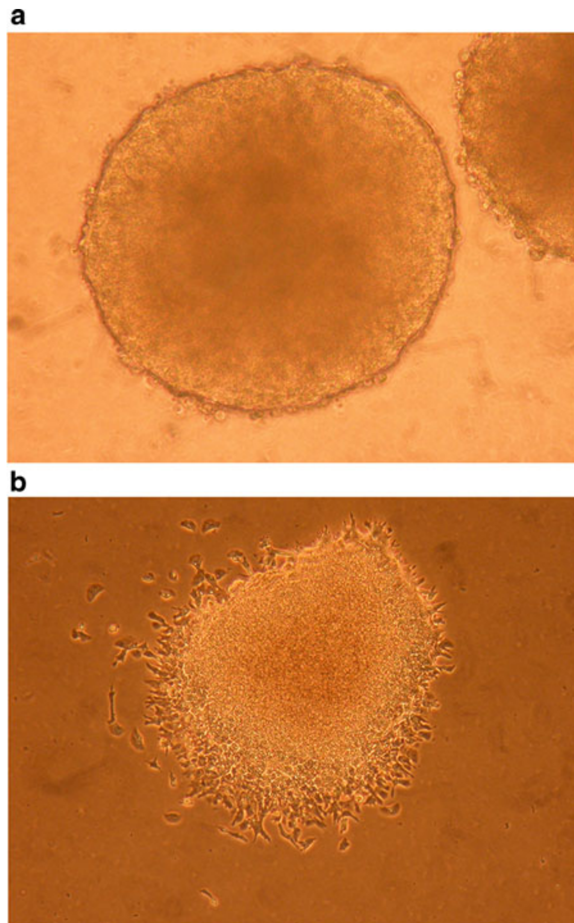

Fig. 2 Images of EBs at different stages of differentiation. A light microscope image of (**a**) a day-4 EB growing in suspension and (**b**) a day-6 EB growing on attached gelatinized tissue culture dish

7. ES cells will aggregate at the bottom of each drop and form EBs. Leave hanging drop dishes undisturbed in the incubator for 3 days.

3.1.6 Growth of EBs in Suspension

1. On day 3, transfer the EBs to bacteriological dishes. Prepare 17 % FBS and add 1 μL RA stock per 100 mL medium for a final concentration of 0.1 μM.
2. Pipette 3 mL of medium with RA onto the lids of the hanging drop dish to wash down the EBs. Collect and transfer the EBs of one hanging drop dish to one bacteriological dish. Wash the lid three times with medium.
3. On the next day (day 4) (Fig. 2a), replace the medium with fresh 17 % FBS medium with 0.1 μM RA (Fig. 1b).

3.1.7 Growth of EBs in Attachment Phase

1. On day 5, EBs are plated on 10 cm tissue culture dish and they remain attached for the rest of the 21-day differentiation period (Fig. 2b).
2. Add 4 mL of 0.1 % gelatin in a 10 cm tissue culture dish, cover the bottom of the dish evenly with gelatin, and aspirate gelatin. Set dish aside for later use.
3. Add 9 mL 17 % FBS medium in each dish, and transfer 25 EBs to each 10 cm dish. Gently move the dish around for even distribution of EBs.
4. On day 6, per mL 17 % FBS medium, add 5 μL of β-glycerophosphate (for a final concentration of 10 mM) and 5 μL of ascorbic acid (for a final concentration of 50 μg/mL). β-Glycerophosphate and ascorbic acid are kept throughout differentiation (Fig. 1b). Change medium every other day.
5. On day 10, dexamethasone (100 nM) is added and kept throughout the differentiation. Add 1 μL dexamethasone per mL medium (Fig. 1b).

3.1.8 Detection of Osteogenic Lineage Cells: von Kossa Stain

von Kossa stain is a routine assay which detects the presence of calcium deposits in culture. Mature osteoblasts secrete calcium and phosphate ions on a matrix composed mainly of collagen 1 and other organic components. The silver ions present in the von Kossa stain displace calcium ions, and the silver ions are subsequently visualized (Fig. 3a).

1. Prepare 10 % neutral formalin buffer solution by adding 100 mL 36.5 % formaldehyde to 16 g Na_2HPO_4, 4 g $NaH_2PO_4 \cdot H_2O$ and 1 L distilled H_2O.
2. Prepare 2.5 % silver nitrate solution by dissolving 2.5 g silver nitrate into distilled H_2O.
3. On day 21, carefully aspirate medium from the dish, wash cells three times with distilled H_2O.
4. Fix cells in 10 % neutral formalin buffer for 2 h.
5. Rinse cells with distilled H_2O three times.
6. Stain cells with 2.5 % silver nitrate solution for 30 min.
7. Aspirate all silver nitrate solution and rinse three times with distilled H_2O. Cells are now ready to be examined for mineral deposits (appears black).

3.1.9 Detection of Osteogenic Lineage Cells: Osteogenic Marker Expression

RNA Isolation and Reverse Transcription

1. RNA is isolated using the Qiagen RNeasy Plus Mini Kit according to the manufacturer's instruction. RNA should be stored at −80 °C (*see* **Notes 3 and 4**).
2. In a 1.5 mL Eppendorf tube, add 4 μL Oligo $(dt)_{18}$ primer, 4 μg RNA, 4 μL 10 mM dNTP, and 36 μL UltraPure Distilled H_2O.
3. Heat mixture to 65 °C for 5 min.

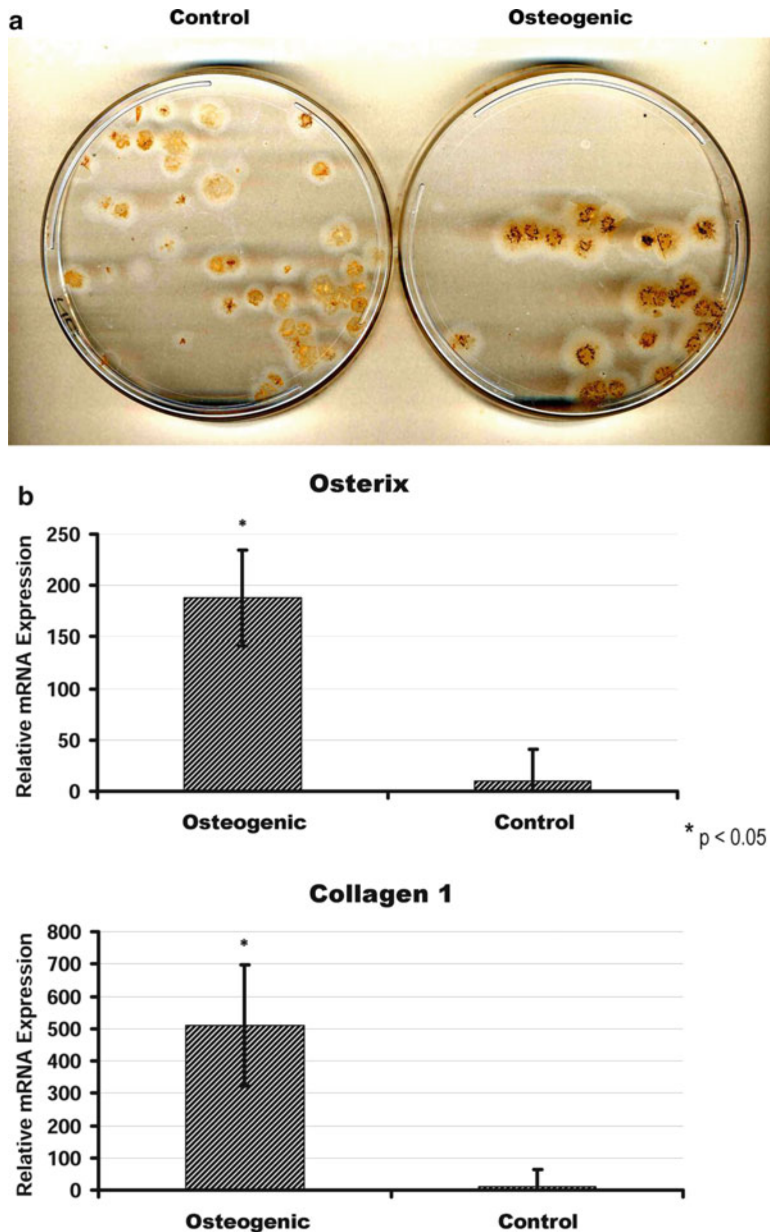

Fig. 3 Detection of osteogenic lineage cells. (**a**) von Kossa stain of differentiated day-21 nodules. Cells grew in the presence of osteogenic differentiation medium (Osteogenic) had more mineral deposition compared to those grew in 17 % FBS medium (Control). (**b**) Expression of bone markers in the nodules detected by Real-Time PCR. Cells cultured in the presence of osteogenic medium (Osteogenic) had higher abundance of Osterix and Collagen 1 compared to those cultured in the presence of only 17 % FBS medium (Control) (experiments were repeated at least three times, data presented are means ± SD)

4. Quickly chill contents on ice.
5. In each tube, add 16 μL 5× first strand buffer, 8 μL 0.1 M DTT, and 4 μL RNaseOUT Recombinant Ribonuclease Inhibitor.

6. Incubate at 42 °C for 2 min.
7. Add 4 μL SuperScript II Reverse Transcriptase to each tube.
8. Incubate at 42 °C for 50 min. Then incubate at 70 °C for 15 min.
9. Store cDNA at −80 °C.

Real-Time PCR

1. Using a 384-well plate, in each well, add 2 μL forward primer stock, 2 μL reverse primer stock, 10 μL SYBR green PCR Master Mix, and 1 μL 10 ng/ μL cDNA. For each sample, do triplicates.
2. Amplify cDNA using the Bio-Rad's CFX384 TouchTM Detection System. Program the system as the following: step 1: 94 °C for 6 min, step 2: 94 °C for 1 min, step 3: 56 °C for 1 min, step 4: 72 °C for 1 min, step 5: go to step 2 45 times, step 6: 72 °C for 7 min, step 7: 4 °C for 5 h.
3. Normalize gene expression to a housekeeping gene (i.e., L32) (Fig. 3b).

4 Notes

1. For all liquids used in tissue culture such as PBS, 0.1 % gelatin, and distilled H_2O, use only after they have been autoclaved.
2. Use autoclaved pipette tips for all work in tissue culture.
3. Use UltraPure RNAse DNAse Distilled H_2O for steps in reverse transcription and real-time PCR.
4. Use sterile, DNase- and RNase-free, filtered pipette tips and DNase- and RNase-free gloves when working with RNA and cDNA.

Acknowledgement

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Limbal Stromal Tissue Specific Stem Cells and Their Differentiation Potential to Corneal Epithelial Cells

Kishore Reddy Katikireddy and Ula V. Jurkunas

Abstract

From the derivation of the first human embryonic stem (hES) cell line to the development of induced pluripotent stem (iPS) cells; it has become evident that tissue specific stem cells are able to differentiate into a specific somatic cell types. The understanding of key processes such as the signaling pathways and the role of the microenvironment in epidermal/epithelial development has provided important clues for the derivation of specific epithelial cell types.

Various differentiation protocols/methods were used to attain specific epithelial cell types. Here, we describe in detail the procedure to follow for isolation of tissue specific stem cells, mimicking their microenvironment to attain stem cell characteristics, and their potential differentiation to corneal epithelial cells.

Keywords: Tissue specific stem cells, Multipotent stem cells, Limbal stromal stem cells, Differentiation, Epithelial progenitor cells, Corneal epithelium, Epidermis

1 Introduction

In the cornea, the maintenance of healthy corneal epithelium is physiologically achieved by limbal stem cells (LSC), which are essential for the integrity and function of corneal epithelium (1). However, chronic ocular surface problems, such as chemical and thermal injuries, result in undermined visual impairment and corneal stem cell dysfunction. Patients with such problems require extensive surgical procedures, which include stem cell transplantation with cultured limbal cells (2). In contrast, bilateral injuries require allogeneic transplantations with immunosuppressive therapy (3). In addition to the successful clinical application of cultured limbal epithelial cells in humans, there are alternative sources for corneal epithelial cells, such as embryonic stem cells (ESc) (4), tissue specific stem cells from limbal stroma (5), and mesenchymal stem cells (6).

Several groups have published on the differentiation of ES (4, 7, 8) cells and pluripotent stem cells to corneal epithelial cells (9). Recently, we have developed novel method for isolation of limbal stromal stem cells and creation of three-dimensional (3D)

culture system that simulates limbal microenvironment and induces stem cell characteristics that induce stromal stem cells differentiation into corneal epithelium (5).

In this chapter, we describe the procedure to induce limbal stromal stem cells to attain stem cell characteristics by developing a three-dimensional (3D) culture system, and to differentiate them to corneal epithelial cell phenotype.

2 Materials

We used ultrapure water as a diluent unless otherwise indicated and sterilized all components using a 0.22 μm filter.

2.1 Cells

1. Human limbal stromal stem cells.
2. Human limbal stromal stem cells derived via ectopic expression of human SSEA4, followed by OCT4, SOX2, Nanog, Rex1, p63, and ABCG2 (5).

The use of human cells is subject to regulatory guidelines specific to each country.

2.2 Reagents

1. Knockout embryonic stem cell/induced pluripotent cell medium (knockout ESC/iPSC) (Life Technologies, cat. no. 10828-028).
2. Fetal bovine serum (FBS) (Life Technologies, cat. no. 10437-077).
3. Nonessential amino acids (Life Technologies, cat. no. 11140-050).
4. Penicillin–streptomycin (Life Technologies, cat. no. 10378-016).
5. L-glutamine (Life Technologies, cat. no. 25030-081).
6. Leukemia inhibitory factor (Life Technologies, cat. no. PHC9481).
7. Matrigel (BD Biosciences, cat no. 354234).
8. Human fibroblast growth factor-basic (bFGF) (Life Technologies, cat. no. PHG0261).
9. Low-glucose, low-calcium Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, cat. no. D6046).
10. Human recombinant bone morphogenetic protein-4 (Sigma-Aldrich, cat. no. SRP3016).
11. All-*trans* retinoic acid (RA) (Sigma-Aldrich, cat. no. R2625).
12. Recombinant human epidermal growth factor (EGF) (Sigma-Aldrich, cat. no. E9644).

13. Hank's balanced salt solution (Life Technologies, cat. no. 14025076).
14. Dispase II (Life Technologies, cat. no. 17105-041).
15. 0.025 % Trypsin–EDTA (Life Technologies, cat. no. R001100).
16. Ham's F12 medium (Sigma-Aldrich, cat. no. 51651C).
17. Hydrocortisone (Sigma-Aldrich, cat. no. H4001).
18. Insulin (Sigma-Aldrich, cat. no. I2643).
19. Adenine (Sigma-Aldrich, cat. no. A2786).
20. 0.22 μm filter (Corning, cat. no. 430049).
21. 70 μm nylon filter (Falcon, cat. no. 352350).
22. SSEA4 (Stage Specific Embryonic Antigen) (Abcam, cat. no. 109884) (for Immunofluorescence staining).
23. SSEA4 (Cl MC-813-70) (R&D systems, cat. no. MAB1435).
24. Goat anti-mouse IgG (Miltenyi Biotech, cat. no. 130-048-402).
25. Oct4 (Octamer-binding Protein 4) (Abcam, cat. no. 109884).
26. TRA-1-60 (Abcam, cat. no. 109884) (*see* **Note 1**).
27. Cytokeratin 12 (L-20) (Santa Cruz, cat. no. SC-17099).
28. Cytokeratin 3 (AE5) (Millipore, cat. no. CBL218).
29. Cytokeratin 8 (E432) (Millipore, cat. no. 04-588).
30. Cytokeratin 14 (C-14) (Santa Cruz, cat. no. SC-17104).
31. Cytokeratin 15 (LHK15) (Santa Cruz, cat. no. SC-47697).
32. β1-Integrin (Santa Cruz, cat. no. SC-9970).
33. α-SMA (B4) (Santa Cruz, cat. no. SC-53142).
34. N-cadherin (H-63) (Santa Cruz, cat. no. SC-7939).
35. E-cadherin (BD Biosciences, cat. no. 610182).
36. Alexa Fluor 488 anti-Mouse IgG (Invitrogen, cat. no. A-21141).
37. Alexa Fluor 647 anti-Rabbit IgG (Invitrogen, cat. no. A-21244).

2.3 Cell Culture Reagents

1. Enriched stem cell medium: 500 ml of Knockout basal embryonic stem cell/induced pluripotent cell medium (knockout ESC/iPSC), supplemented with the following reagents with final concentrations as follows: 1 % nonessential amino acids, 1 % penicillin–streptomycin, 1 % L-glutamine, 10 ng/ml Leukemia inhibitory factor (LIF), and 4 ng/ml Human Fibroblast Growth Factor-Basic (bFGF) under sterile conditions (*see* **Note 2**).

2. Induction medium: Dulbecco's Modified Eagle's Medium (DMEM) without pyruvate, with final concentrations of 25 ng/ml human recombinant bone morphogenetic protein-4 (BMP-4), 1 μM all-*trans* retinoic acid (RA), and 10 ng/ml recombinant human epidermal growth factor (EGF).
3. Differentiation medium: Three parts low-glucose DMEM with pyruvate and one part Ham's F12 medium (DMEM:Ham's F12; 3:1), 5 % FBS, 1 % penicillin–streptomycin, 500 ng/ml hydrocortisone, 5 μg/ml insulin, 10 ng/ml adenine, and 10 ng/ml recombinant human EGF. All medium were filter-sterilized using a 0.22-μm filter and stored at 4 °C.
4. Preparation of Matrigel coated plates: Matrigel was diluted in cold DMEM at different concentrations (i.e., thicknesses). For a thin-coating, 200 μl/cm^2 of a 5 % Matrigel solution was added to culture plates, and for the 3D culture system, 200 μl/cm^2 of 50 % Matrigel solution was added and incubated at 37 °C/5 % CO_2 for 1 h (*see* **Note 3**).

3 Methods

3.1 Isolation and Sorting of Human Limbal Stromal Stem Cells

1. Human limbal stromal stem cells isolated from human corneoscleral rims.
2. Corneoscleral tissues are rinsed with Hank's balanced salt solution; the sclera, conjunctiva, iris, trabecular meshwork, and corneal endothelium are removed.
3. Then the limbal tissue is cut into quadrants and placed in 1.2 IU of Dispase II for 1 h at 37 °C (*see* **Note 4**).
4. Intact epithelial sheets are removed from stroma and the de-epithelialized stroma further digested with 0.025 % Trypsin–EDTA for 30 min at 37 °C to yield single cells.
5. Stromal cell suspension is then filtered with 70 μm nylon filter, and cells are resuspended in KnockOut ESC/iPSC medium, seeded on thinly coated Matrigel 6-well plates, and allowed to grow for 12–15 days (*see* **Note 5**) (Fig. 1).
6. After enrichment with stem cell medium for 12 days, the limbal stem cells are treated with 0.025 % Trypsin–EDTA, resuspended in PBS at a concentration of 5 × 10^4 cells/μl, and the suspension incubated with 1 μl of anti-SSEA4 antibody for 30 min at 4 °C.
7. Cells are washed twice with PBS to remove unbound antibody and the cell suspension incubated with 10 μl of magnetic beads tagged with SSEA4 for 20 min at 4 °C.
8. The cells are separated using magnetic-activated cell sorting columns.

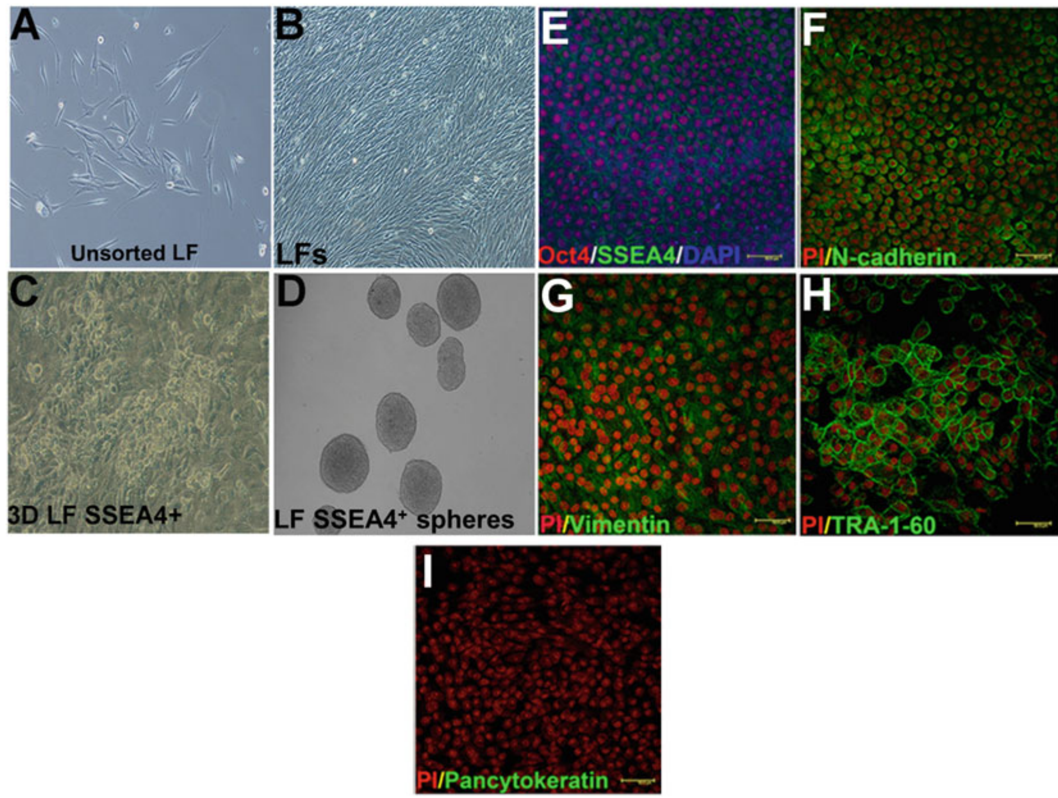

Fig. 1 Culture of LF cells. (**a**) Cultured human LFs at day 6; (**b**) LFs near confluence at day 15; (**c**) Sorted SSEA4$^+$ expressing LF cells in 3D culture showing a round compact morphology; (**d**) LF SSEA4$^+$ spheres; (**e**) LF SSEA4$^+$ cells positive for SSEA4 (*green*), Oct4 (*red*), (**f**) N-cadherin (*green*), (**g**) vimentin (*green*), (**h**) TRA-1-60 (*green*), and (**i**) negative for the epithelial marker Pancytokeratin (PCK). Jurkunas et al, 2014; reproduced by copyright permission of the Wiley Publication

9. The SSEA4$^+$ fractions of limbal stromal stem cells are re-plated every 5–6 days with KnockOut ESC/iPSC in 3D culture to enrich stem cell niche.
10. SSEA4$^+$ cells in 3D cultures showed a round compact morphology, and stained positively for stem cell markers (Fig. 1).

3.2 Sphere Formation, Induction, and Differentiation of SSEA4$^+$ Limbal Stromal Stem Cells

1. SSEA4$^+$ limbal stromal stem cells in a single-cell suspension are seeded onto polystyrene petri dishes at a density of $5 \times 10^4/\text{cm}^2$ with KnockOut ESC/iPSC medium without serum (*see* **Note 6**) (Fig. 1).
2. At day 7, spheres are treated with induction medium consisting of RA, BMP-4 and EGF for 4 days at 37 °C in 5 % CO_2 (*see* **Note 7**).
3. After induction, spheres are dissociated into a single cell suspension by treating them with 0.025 % Trypsin–EDTA for 10 min.

4. The cells are then centrifuged, suspended in differentiation medium, and seeded onto thin-coated Matrigel culture inserts at a density of 1,000–1,200 cells/cm^2 and incubated at 37 °C in 5 % CO_2 for 9 days.
5. During differentiation, SSEA4$^+$ cells initially exhibited a fibroblast-like appearance without a significant difference in morphology (*see* **Note 8**).
6. By day 4 of differentiation, increased numbers of round and cobblestone-appearing cells with an epithelial morphology can be found; by day 9, cells become positive for corneal epithelial markers (Fig. 2).

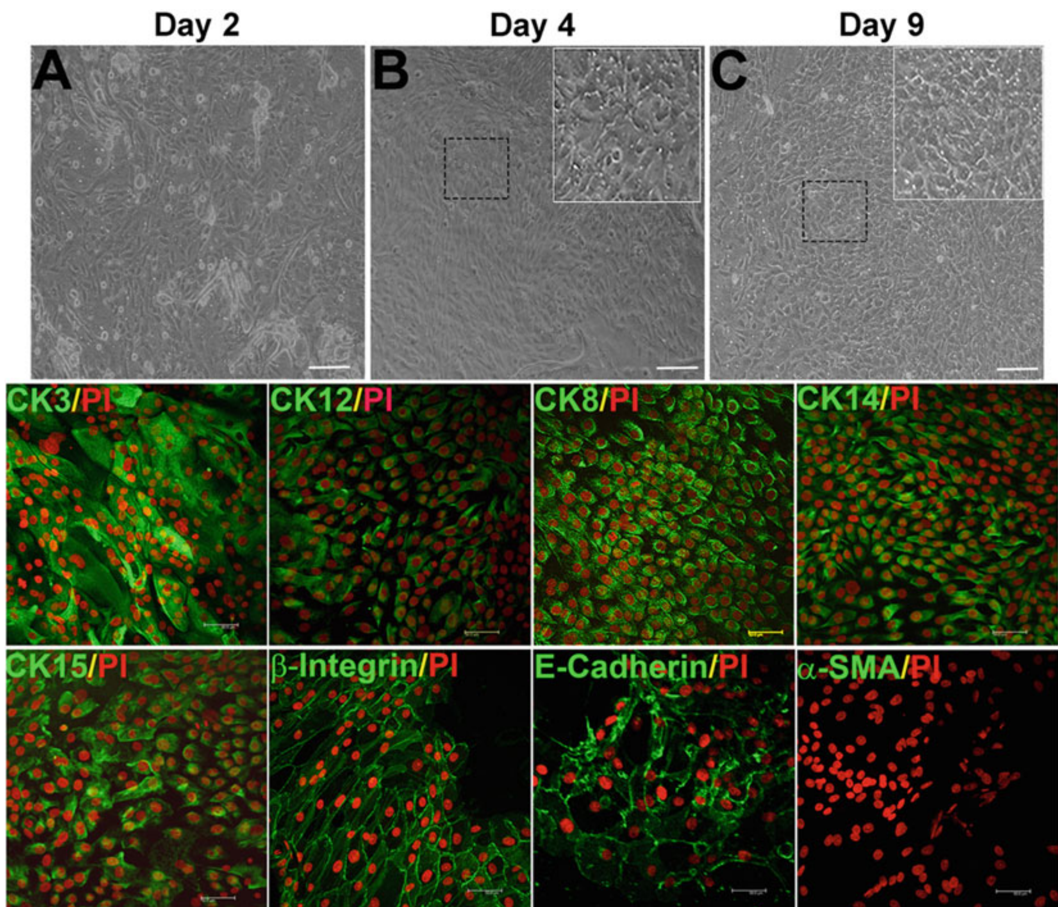

Fig. 2 Differentiation of LF SSEA4$^+$ cells. (**a**) LF SSEA4$^+$ cells at day 2; (**b**) LF SSEA4$^+$ cells at day 4, cells appear to be round and compact (*inset*); (**c**) LF SSEA4$^+$ cells appear epithelia-like at day 9 (*inset*). *Down panel* differentiated LF SSEA4$^+$ cells are positive for corneal epithelial markers CK3, CK12, CK8, CK14, CK15, β1 integrin, and E-cadherin (all *green*), and negative for α-SMA PI nuclear stain. Jurkunas et al, 2014; reproduced by copyright permission of the Wiley Publication

4 Notes

1. Stem cell markers such as SSEA4, Oct4, and TRA-1-60 are purchased from Millipore (Human Embryonic Stem Cell Marker Panel, Cat. no. ab109884).
2. Stock solutions and prepared medium are stored at −20 °C, and must be used within a month after preparation. The required volume of induction/differentiation medium is pre-warmed at room temperature until they are no longer cool to the touch.
3. Dilution instructions are provided with each lot of Matrigel. Matrigel (5 ml vial) is thawed overnight at 4 °C. Cryovials, conical tubes, and pipette tips for dilution are prechilled prior to dilution of Matrigel. Using chilled pipette tips, an aliquot of Matrigel is added to cold DMEM, and immediately distributed into the tissue culture dishes. The Matrigel-coated plates are stored in a refrigerator at 4 °C overnight prior to being used and can be stored for up to 1 week.
4. After 30 min incubation, check the tissue under the microscope for possible epithelial cell dislodging.
5. Depending on cell yield, cells can be seen between days 7–8 with slow proliferation.
6. Sphere formation can be noted within 48 h, and can be monitored with phase-contrast microscopy up to 7 days in culture.
7. At this stage, the cells should be checked for their conversion to an ectodermal cell linage, either by immunofluorescence or RT-PCR using ectodermal cell lineage markers such as CK8, and pan-cytokeratins.
8. During the differentiation step, cells can be monitored on daily basis for typical epithelial cell morphology, which is usually expressed between days 4 and 9. The time frame for the appearance of typical epithelial cell morphology is also dependent on cell density when seeding for the differentiation step. No more than 1,000–1,200 cells/cm^2 should be seeded in each well. A higher cell number may cause more cell death during the differentiation process.

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Erratum to: Resolving Heterogeneity: Fluorescence-Activated Cell Sorting of Dynamic Cell Populations from Feeder-Free Mouse Embryonic Stem Cell Culture

Jurriaan Hölzenspies, Gelo Dela Cruz, and Joshua M. Brickman

Erratum to: Methods in Molecular Biology
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There is an error in given name and family name of the author Jurriaan Hölzenspies. The correct name should read as Jurriaan J. Hölzenspies (given name: Jurriaan, given name: J, and family name: Hölzenspies)

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