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Andras Nagy
Kursad Turksen *Editors*

Patient-Specific Induced Pluripotent Stem Cell Models

Generation and Characterization

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Preface

Rapid developments in the field of induced pluripotent stem (iPS) cells have provided novel opportunities and approaches, both for better understanding a number of human diseases and for developing new platforms for drug development and screening for such diseases. To complement our volume on methods for establishing iPS cells, we have also collected representative protocols on various disease models. We are grateful to all the contributors who shared the details of their protocols for this volume; without their generous efforts, this volume would not have been possible.

Dr. John Walker, Editor in Chief of *Methods in Molecular Biology*, was instrumental in getting this volume off the ground, and we thank him for it. Patrick Marton, Editor of Springer Protocols, was also very supportive as we put together this volume. We also thank David Casey and Monica Suchy for helping us to correct missing details during book production.

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Generation of Patient-Specific induced Pluripotent Stem Cell from Peripheral Blood Mononuclear Cells by Sendai Reprogramming Vectors

Oscar Quintana-Bustamante and Jose C. Segovia

Abstract

Induced pluripotent stem cells (iPSC) technology has changed preclinical research since their generation was described by Shinya Yamanaka in 2006. iPSCs are derived from somatic cells after being reprogrammed back to an embryonic state by specific combination of reprogramming factors. These reprogrammed cells resemble all the characteristic of embryonic stem cells (ESC). The reprogramming technology is even more valuable to research diseases biology and treatment by opening gene and cell therapies in own patient's iPSC. Patient-specific iPSC can be generated from a large variety of patient cells by any of the myriad of reprogramming platforms described. Here, we describe the generation of patient-specific iPSC from patient peripheral blood mononuclear cells by Sendai Reprogramming vectors.

Keywords: Patient-Specific induced Pluripotent Stem Cell, Peripheral blood mononuclear cells, Sendai vectors, Reprogramming, Pluripotency, Self-renewal

1 Introduction

The generation of induced pluripotent stem cells (iPSC) described by Shinya Yamanaka (1–3) has meant a new stage in biomedicine. Although somatic cells can be reprogrammed back to an embryonic state by cell fusion or nuclear transfer since long time ago, only few laboratories had cell reprogramming technology applied to a limit number of cell types from a reduce variety of species. However, when Shinya Yamanaka described for the first time a simple method to reprogram mouse (1, 2) and human fibroblasts (2) back to a pluripotent state by the delivery of four embryonic transcription factors (OCT4, SOX2, KLF4, and cMYC), cell reprogramming was broadly opened to the research community. This newly described technology was widely expanded by its high reproducibility and easy implementation. Quickly, the original iPSC technology was modified regarding combinations of reprogramming factors (4–6), their delivery method (7–12), or the cell type to be reprogrammed (3, 13–16). More importantly, iPSC platforms were used to reprogram back somatic cells from patients of numerous genetic diseases (17).

These patient-specific iPSCs show self-renewal and pluripotency similar to embryonic stem cells (ESC), which are leading up the development of new autologous cell and gene therapy approaches (17) and compelling disease models (18). Here, we described a simple method to generate patient-specific iPSC from peripheral blood mononuclear cells (PB-MNC) by Sendai Reprogramming vectors (SeV). PB-MNC means a noninvasive, easily accessible, and routine somatic cell source in the clinic. SeV implies an integration-free and self-erasable reprogramming system, since these viruses are no genotoxic, are able to infect a wide range of cell types with high transduction efficiency, and are eliminated from the cell once reprogrammed because of their lower replication rate compared to the iPSC one. We have successfully reprogrammed PB-MNC samples from healthy donors and from anemic patients, indicating the efficacy of the reprogramming protocol described here.

2 Materials

All the procedures described here are based on our feeder-dependent iPSC culture; however, this system can be adapted to other iPSC culture conditions.

2.1 General Equipment

1. Biohazard safety cabinet certified for handling of biological materials.
2. Incubator set at 37 °C with 5 % CO_2 and 95 % humidity.
3. Standard light microscope for cell counting.
4. Stereomicroscope equipped with a zoom from 1× to 6.3× and thermo plate able to heat at 37 °C. The stereomicroscope has to be placed in a biological safety cabinet.
5. Laboratory centrifuge.
6. Vortex.
7. Laboratory water bath.
8. Pipette-aid.
9. Micropipettors.
10. Stripper (MidAtlantic Diagnostics, Inc., Marlton, NJ, USA) equipped with 200 μm stripper tips.
11. Neubauer hemocytometer.
12. 15 mL conical tubes.
13. 50 mL conical tubes.
14. Tissue culture treated 6-well plates.
15. Tissue culture treated 24-well plates.
16. Tissue culture treated p100 plates.

17. Tissue culture treated p150 plates.
18. Cryotubes.
19. Mr. Frosty Freezing container (Thermo Fisher Scientific, Waltham, MA, USA).
20. Cryogenic tank.

2.2 Peripheral Blood Mononuclear Cell

1. PBE buffer: Dulbecco's phosphate buffered saline without calcium chloride and magnesium chloride (PBS, Sigma-Aldrich, St. Louis, MO, USA)/0.5 % v/v bovine serum albumin (BSA, Sigma-Aldrich)/2 mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich). Filtered through a 0.22 μm filter.
2. Ficoll-Paque Plus (density 1.077, GE Healthcare Life Science, Madrid, Spain).
3. Türk's solution: 1 mL of 1 % w/v aqueous methylene blue solution (Sigma-Aldrich)/2 % acetic acid in water.
4. Trypan Blue Solution (Life Technologies, Madrid, Spain).
5. Dimethyl sulfoxide (DMSO, Sigma-Aldrich).
6. Hyclone Fetal Bovine Serum (FBS, GE Healthcare Life Science).
7. Penicillin-Streptomycin (PS, Life Technologies).

2.3 SeV Reprogramming

1. StemSpan™ SFEM (STEMCELL Technologies, Vancouver, Canada).
2. Human Stem Cell Factor (hSCF, EuroBiosciences, Friesoythe, Germany).
3. Human Fms-related tyrosine kinase 3 ligand (hFLT3L, EuroBiosciences).
4. Human thrombopoietin (hTPO, EuroBiosciences).
5. Human granulocyte colony-stimulating factor (G-CSF, Neulasta, Amgen, Barcelona, Spain).
6. Human interleukin 3 (hIL3, EuroBiosciences).
7. PB-MNC medium: 0.22 μm filtered StemSpan™ SFEM/100 ng/mL hSCF/100 ng/mL hFLT3L/20 ng/mL hTPO/10 ng/mL G-CSF/2 ng/mL hIL3/0.5 % PS.
8. CytoTune®-iPS Sendai Reprogramming Kit (Life Technologies).

2.4 Human-Induced Pluripotent Cell Culture

1. Dulbecco's Modified Eagle Medium (DMEM, Life Technologies).
2. hFGF-basic (154 aa) (Peprotech, Rocky Hill, NJ, USA).
3. Knockout™ DMEM medium (KO-DMEM, Life Technologies).
4. MEM Nonessential Amino Acids Solution (NEAA, Life Technologies).

5. GlutaMAX™ supplement (Life Technologies).
6. Knockout™ Serum Replacement (KO-SR, Life Technologies).
7. 2-Mercaptoethanol (Life Technologies).
8. Gelatin solution: 0.22 μm filtered 0.1 % gelatin (Sigma-Aldrich) in water.
9. TrypLE™ Select (Life Technologies).
10. Feeder medium: 0.22 μm filtered DMEM/20 % FBS/1 % GlutaMAX/1 % NEAA/1 % PS.
11. hES medium: 0.22 μm filtered KO-DMEM/1 % NEEA/1 % GlutaMAX/0.5 % PS/20 % KO-SR/50 μM 2-mercaptoethanol/10 ng/mL hFGF-basic.
12. Human foreskin fibroblast (HFF-1, ATCC, Barcelona, Spain).
13. Mouse embryonic fibroblast CF-1 (MEF, ATCC).
14. Collagenase IV solution: dissolve 10 mg/mL of Collagenase IV (Life Technologies) in KO-DMEM. Dilute this first solution to 1 mg/mL Collagenase IV and filter it by 0.22 μm filter. Collagenase IV concentration can be kept at −20 °C; however, they lose activity very quickly at 4 °C.
15. Y-27632 (Rock Inhibitor, Reagents Direct, Encinitas, CA).

3 Methods

All procedures for cell processing should be performed using sterile technique and universal handling precautions in a biological safety cabinet. ESC/iPSC culture skills are required.

3.1 Peripheral Blood Mononuclear Cell Purification

1. Collect 5–10 mL peripheral blood (PB) by standard procedures. Keep it at room temperature in the presence of an anticoagulant.
2. Mix the PB with twice the volume of PBE buffer.
3. Add 5 mL of Ficoll-Paque Plus to a 15 mL conical tube (*see* **Note 1**).
4. Slowly layer 10 mL of PB on top of the Ficoll-Paque Plus (*see* **Note 2**) (Fig. 1). Avoid disturbing the two layers (*see* **Note 3**).
5. Centrifuge at 400 × *g* for 25 min at room temperature with the centrifuge brake off.
6. The sample will be separated in different layers by a density gradient (Fig. 1). Handle the tube very carefully not to alter the layer distribution.
7. Remove the plasma upper layer by a sterile pipette. Care not to disturb the white layer of PB-MNC underneath. Keep part of this plasma to analyze mycoplasma contamination in the sample (*see* **Note 4**).

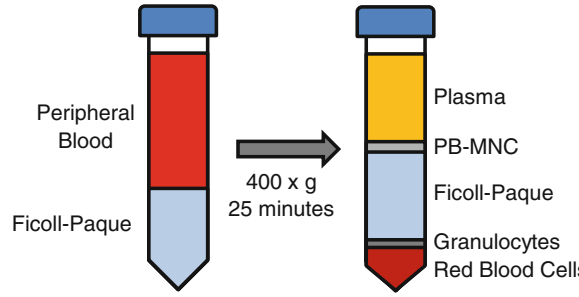

Fig. 1 Diagram shows the layer distribution before and after Ficoll-Paque gradient is formed by centrifugation. Different cell layers are indicated

8. Take off thoroughly with the tip of a pipette 5 mL any cell clump stuck in the tube wall in the PB-MNC layer.
9. Remove the PB-MNC layer and transfer it to a 15 mL conical tube.
10. Wash this cell suspension by adding enough PBE volume to dilute any Ficoll-Paque Plus still present. Centrifuge at 300 × *g* at room temperature for 7 min with brake on.
11. Discard the supernatant without disrupting the PB-MNC pellet.
12. Resuspend the PB-MNC pellet by adding 1 mL of PBE.
13. Dilute a small aliquot of cell suspension with Türk's solution (*see* **Note 5**). Count it in the Neubauer hemacytometer.
14. PB-MNC can be used freshly in the next step or can be frozen (*see* **Note 6**).

3.2 Irradiated HFF-1 (irHFF-1) or Irradiated MEF (irMEF) Preparation

1. Grow primary fibroblast in gelatin-coated p150 plate with feeder medium (25 mL/p150 plate).
2. When the feeder is close to confluence, wash it with PBS (Sigma-Aldrich) and detach the cells from the plate by adding 10 mL of TrypLE™ Select/plate and treatment at 37 °C for 5–7 min.
3. Collect the cells in a 15 mL conical and wash the plate with 5 mL of PBS/5 % FBS. Centrifuge the cell suspension at 300 × *g* for 7 min.
4. Resuspend the cells in feeder medium and seed into new gelatin-coated p150 at 1.7×10^4 cell/cm^2 cell density with 22–25 mL feeder medium.
5. Expand the primary feeders up to four to five passage. To irradiate the primary fibroblasts, wash the cells with PBS and treat by TrypLE™ Select to detach them.

6. Collect the cells in 50 mL conical tube and centrifuge. Resuspend the cells in fresh feeder medium.
7. Irradiate the cell suspension with 45 Gy using a Philips MG324 X-ray equipment (Philips, Hamburg, Germany) set at 300 kV, 10 mA, delivering a dose rate of 1.03 Gy/min.
8. Centrifuge the irradiated fibroblasts at 300 × *g* for 7 min. Resuspend in feeder medium and count cell concentration. Adjust cell concentration to get 8×10^6 cells/mL.
9. Distribute 4×10^6 irradiated fibroblasts per each cryotube. Add 500 μL of chill 20 % DMSO/80 % FBS in each cryotube. Keep all the cryotubes in a Mr. Frosty Freezing container at −80 °C. After 24–48 h, transfer the cryotubes to a cryogenic tank.

3.3 Plate Coating by Irradiated Feeder

1. Cover the plate surface with gelatin solution, and keep at 37 °C for at least 30 min.
2. Thaw an irradiated fibroblast cryotube in a 37 °C water bath. Transfer thawed cell to 15 mL conical tube. Dilute cell suspension by adding 10 mL of feeder medium. Centrifuge at 300 × *g* for 7 min. Resuspend the cells in feeder medium. Count by treating with Trypan Blue to exclude dead cells.
3. Remove gelatin solution from the 37 °C warm plate and add feeder medium containing the cells. Add enough volume of cell suspension to the plate to get $0.7–1.5 \times 10^4$ cell/cm^2. Allow cells to attach for at least 12 h. Keep at 37 °C until using.
4. Before adding hiPSC, wash the plate with KO-DMEM. Discard nonused feeder-coated plates after 4 days to be seeded.

3.4 Generation of PB-MNC hiPSC by SeV

1. Culture PB-MNC up to 5×10^5 cells per mL in PB-MNC medium (*see* **Notes 7** and **8**).
2. Keep the cells at 37 °C for 4 days (*see* **Note 9**).
3. Transfer the cell suspension into a 15 mL conical tube. Wash the plate thoroughly with StemSpan™ SFEM to collect the remained cells.
4. Centrifuge cells at 300 × *g* at room temperature for 7 min.
5. Aspirate supernatant (being careful not to disturb the cell pellet).
6. Dilute the cell pellet in StemSpan™ SFEM/0.5 % PS.
7. Count by Türk's or Trypan Blue solution.
8. Adjust the cell concentration to 1×10^6 cells per mL in PB-MNC medium.

9. Mix SeV from CytoTune®-iPS Sendai Reprogramming Kit in a same volume of PB-MNC medium as the cell suspension volume (*see* **Note 10**).
10. Keep the cells at 37 °C overnight.
11. Wash the cells by transferring to a 15 mL conical tube and spinning down at 300 × *g* at room temperature for 7 min.
12. Resuspend the infected PB-MNC in PB-MNC medium at 5×10^5 cells per mL.
13. Keep the cells at 37 °C for 4 days.
14. Observe the cells dairy (Fig. 2) (*see* **Note 11**).
15. Supplement the medium with 10 ng/mL hFGF-basic after 4 days of infection.

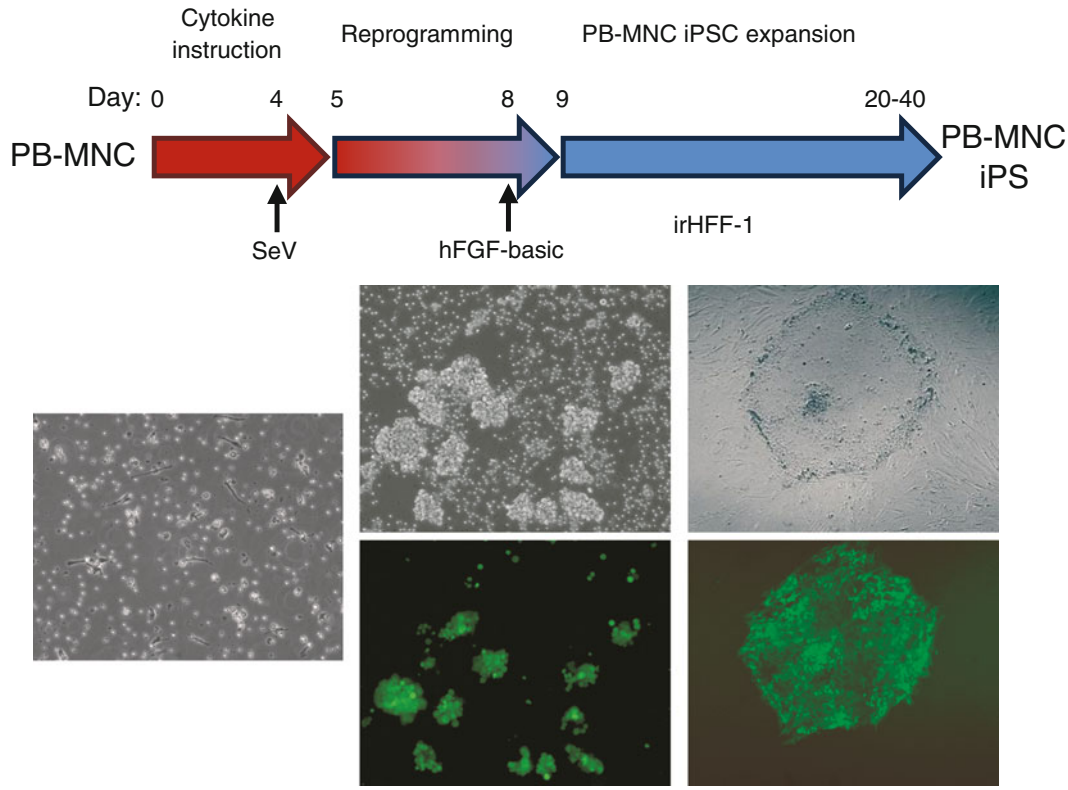

Fig. 2 Scheme of the PB-MNC reprogramming by SeV. Representative images of each stage are showed. Initial heterogeneous population of PB-MNC are instructed by a specific cytokine combination (*left panel*). Reprogramming process, where the SeV-infected PB-MNCs proliferate and change their morphology (*middle panel*). Complete PB-MNC iPSC clone generated by SeV reprogramming (*right panel*). Azami Green SeV was added to the SeV reprogramming cocktail to be able to track the reprogramming process. All the pictures were taken at 100× magnification

16. Next day, collect the cells and transfer to 15 mL conical tube,-centrifuge at 200 × *g* at room temperature for 5 min (*see* **Note 12**).
17. Resuspend the cells in hES medium.
18. Transfer 5×10^4 SeV-infected PB-MNC to an irHFF-coated p100 plate and add up to 10 mL of hES medium (*see* **Note 13**).
19. Replace hES medium every 2 days for warm fresh hES medium; avoid to disturb the cells.
20. Some hES-like colonies will appear on the irHFF. Pick these hES-like colonies under the stereomicroscope by the stripper and transfer the colony fragments to an irHFF-coated p24 well with hES medium (*see* **Notes 14** and **15**).

3.5 PB-MNC hiPSC Culture

1. Passage these PB-MNC hiPS clones every 5–7 days using conventional hES procedures:
2. Manual picking: under the stereomicroscope break up the colonies by the stripper tip or by a 10 μL tip. Transfer to a 15 mL conical tube and centrifuge at 200 × *g* for 2 min. Discard the supernatant carefully without disturbing the cell pellet. Resuspend the cells with fresh hES medium, mix by a soft twice to three times pipetting. Transfer to a new irHFF-1-coated plate.
3. Collagenase IV treatment: wash the hiPSC culture by KO-DMEM. Incubate for 5 min at 37 °C with Collagenase IV solution. Pick up the colonies by stripper tip or by a 10 μL tip under the stereomicroscope. Transfer to a 15 mL conical tube and centrifuge at 200 × *g* for 2 min. Discard the supernatant carefully without disrupting the cell pellet. Resuspend the cells with fresh hES medium, mix by a soft pipetting for five to six times to divide the colonies in a small fragments without breaking up them to a single cell suspension. Transfer to a new irHFF-1-coated plate.
4. Expand each PB-MNC iPSC clone independently (*see* **Notes 16–18**).
5. Cryopreserve part of each PB-MNC iPSC clone when there are enough number of iPSC colonies (around 15–20 colonies). To cryopreserve iPSC, the culture has to be pretreated by hES medium supplemented by 10 μM Rock inhibitor for at least 1 h. Then, break up the colonies in large fragments. Collect the iPSC fragments in a 15 mL conical tube. Spin down at 200 × *g* for 2 min. Discard the supernatant. Resuspend in 500 μL of chill FBS and transfer to a cryotube. Mix in the cryotube with 500 μL of freshly prepared 20 % FBS/80 % DMSO. Keep for 24 h at −80 °C. Transfer for a long storage to a cryogenic tank (*see* **Note 19**).

6. The rest of each PB-MNC iPSC clone will be expanded by further pluripotency characterization and SeV clearance (*see* **Notes 20** and **21**).

4 Notes

1. Ficoll-Paque Plus should be warmed to room temperature before using.
2. Different PB volumes can be used while PB:Ficoll-Paque Plus ratio will be kept.
3. If the layers are disturbed, different layers will not form after centrifugation.
4. Mycoplasma contamination in a sample avoids iPSC generation. Everything involved in iPSC generation/culture must be mycoplasma-free.
5. The acetic acid present in the Türk's solution destroys red blood cells (RBC) from the PB-MNC suspension, facilitating the cell counting.
6. Hematopoietic cells are frozen by diluting 1:1 the cell suspension with 20 % DMSO/80 % Hyclone FBS at 2×10^6–2×10^7 cell per 1 mL cryotube. The cryotubes have to be kept in a Mr. Frosty Freezing (Thermo Scientific) container at −80 °C for 24 h and then transfer to a cryogenic tank.
7. Cytokine combination is a key factor to lead reprogramming from a specific hematopoietic lineage (19). The cytokine combination described here promotes cell reprogramming by SeV from hematopoietic progenitors and/or myeloid cells, avoiding T or B lymphocytes. Other cytokines can be tested to address reprogramming of different cell types.
8. PB-MNC should be placed in the central wells of the plate, and surrounding wells must be filled with PBS or water to avoid the evaporation of PB-MNC medium.
9. Either massive cell death or cell proliferation can be observed. Washing of cells is recommended when cell death occurs or addition of fresh medium to maintain cell concentration when proliferation happens.
10. SeV must be kept on dry ice until addition to the medium. Avoid repeated freezing and thawing of SeV. The multiplicity of infection (MOI) of each SeV carrying each Yamanaka's factor should be 3, meaning three SeV infectious particles of each reprogramming factor per cell. Additionally a SeV coding for a reporter gene (i.e., Azami Green) can be included to visualize the percentage of transduction.

11. 24–48 h post SeV infection, some fluorescent green cells will appear if Azami Green SeV was added to the reprogramming SeV cocktail. During these 4 days after SeV infection, morphological changes will be observed, such as the presence of highly proliferative large cells (Fig. 2).
12. Since some cells are being reprogrammed, the centrifugation speed must be reduced to avoid the killing of these fragile cells.
13. Irradiated feeder must be prepared the day before. irHFF-1 or irMEF can be used. Before adding the cells, feeder density has to be checked, and feeder medium must be washed by KO-DMEM.
14. The first hES-like colonies will appear around day 5–7 post infection; however, these colonies are usually unstable, and they are not able to be expanded as hES-like cells. The more stable and with correct morphology, hES-like colonies will appear 2 weeks after SeV infection.
15. In our experience, when SeV reprogramming is successful, one hiPSC-derived PB-MNC was generated per 6,000 PB-MNCs.
16. We were able to maintain around one sixth of the picked colonies when SeV reprogramming was successful.
17. The PB-MNC iPSC culture has to reach a 50–60 % confluence, and then the cells can be split from one plate to four to six plates.
18. To expand hiPSC quickly, Rock inhibitor can be added to the hES medium in each passage for few passages, because if iPSCs get used to Rock inhibitor, they lessen their properties.
19. To thaw the frozen iPSC clones, introduce the frozen cryovial in a 37 °C water bath. Transfer the cell suspension to a 15 mL conical tube and add slowly 10 mL of hES medium. Spin down at 200 × *g* for 2 min. Resuspend the cell softly with hES medium/10 μM Rock inhibitor, and seed the hiPSC in a new HFF-1-coated 6-well plate with hES medium/10 μM Rock inhibitor. Keep at 37 °C for 48 h without disturbing. Change the medium daily.
20. Standard pluripotency test will consist in analysis of embryonic gene expression, such as OCT4, NANOG, SSEA4, Tra-1-60, SOX2, cMYC, and REX-1, by qRT-PCR and immunofluorescence, karyotype, embryo body, and teratoma formation.
21. SeV clearance will be assessed by disappearance of Azami Green fluorescence, when Azami Green SeV is added to the SeV reprogramming cocktail, and by RT-PCR using specific primers (SeV F *GGATCACTAGGTGATATCGAGC* and SeV R *ACCAGACAAGAGTTTAAGAGATATGTATC*).

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A Doxycycline-Inducible System for Genetic Correction of iPSC Disease Models

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Abstract

Patient-derived induced pluripotent stem cells (iPSCs) are valuable tools for the study of developmental biology and disease modeling. In both applications, genetic correction of patient iPSCs is a powerful method to understand the specific contribution of a gene(s) in development or diseased state(s). Here, we describe a protocol for the targeted integration of a doxycycline-inducible transgene expression system in a safe harbor site in iPSCs. Our gene targeting strategy uses zinc finger nucleases (ZFNs) to enhance homologous recombination at the AAVS1 safe harbor locus, thus increasing the efficiency of the site-specific integration of the two targeting vectors that make up the doxycycline-inducible system. Importantly, the use of dual-drug selection in our system increases the efficiency of positive selection for double-targeted clones to >50 %, permitting a less laborious screening process. If desired, this protocol can also be adapted to allow the use of tissue-specific promoters to drive gene expression instead of the doxycycline-inducible promoter (TRE). Additionally, this protocol is also compatible with the use of Transcription-Activator-Like Effector Nucleases (TALENs) or Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 system in place of ZFNs.

Keywords: Genetic correction, Disease modeling, Homologous recombination, Doxycycline-inducible expression, Gene targeting, Zinc finger nucleases

1 Introduction

In the characterization of iPSC disease models, a commonly used strategy is to genetically manipulate the patient-derived iPSCs to correct the disease phenotype, thus ascertaining the role of a specific gene in the disease. One way to genetically correct defects in iPSC-based disease models is by the site-specific targeting of transgenes into a safe harbor locus. In human iPSCs, the AAVS1 gene has been identified as a putative safe harbor locus (1–4), where we have expressed a number of transgenes (5, 6).

This chapter describes a protocol for the generation of iPSC lines with a doxycycline-inducible transgene expression system targeted into both alleles of the AAVS1 safe harbor locus. To enhance

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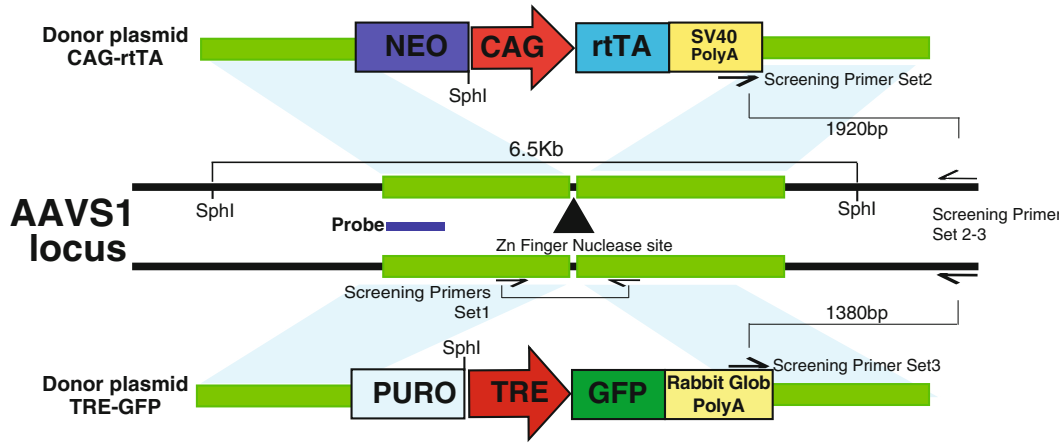

Fig. 1 Design of targeting vectors. ZFNs specific to the AAVS1 locus will cut the DNA in intron 1 of AAVS1. Integration of both targeting vectors will confer dual drug resistance to ESCs/iPSCs. The location of PCR screening primers and Southern blot probe is indicated

Table 1
Percentage of selected clones that are correctly double-targeted from three independent transfections

Transfection attempt	Number of clones screened	Number of double-targeted clones	Percentage of double-targeted clones (%)
1	12	8	67
2	6	5	83
3	10	5	50
Overall	28	18	64

homologous recombination (7, 8), a pair of ZFNs are used to induce a double-stranded break specifically in the AAVS1 locus. The two targeting vectors with arms of homology to AAVS1 express reverse tet activator (rtTA) driven by a constitutive promoter and a tet-response element (TRE) driving the gene of interest (Fig. 1). If knock down of a gene is desired, short hairpins directed against the gene of interest can be cloned downstream of the TRE in the targeting construct. The two targeting vectors are designed to confer dual-drug resistance to double-targeted clones. Clones are screened using PCR to confirm the integration of the targeting vectors and Southern blotting to identify the presence of off-target integration. With the use of dual drug selection, on average >50 % of selected clones are correctly double-targeted (Table 1). The entire process takes about 3 weeks, excluding the time needed for the clones to expand (Fig. 2). This protocol can

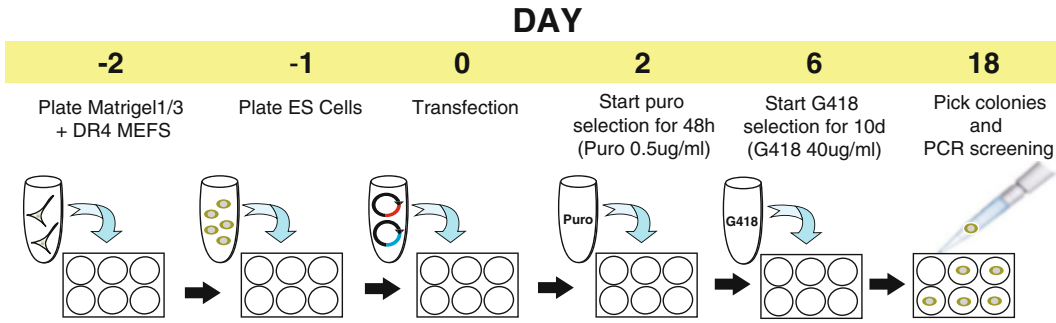

Fig. 2 Schematic outlining the processes involved in the generation of a doxycycline-inducible transgene expression system in hESCs/iPSCs

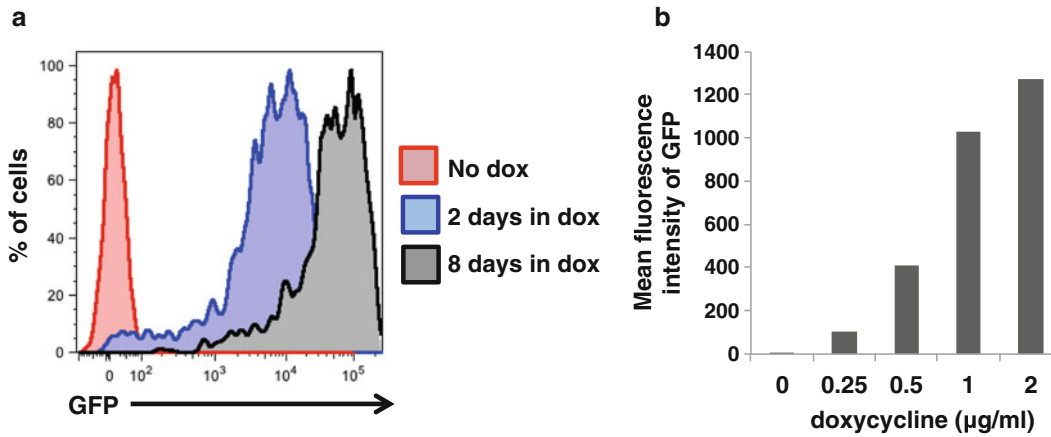

Fig. 3 GFP expression in hematopoietic progenitors differentiated from hESC line with doxycycline-inducible GFP expression under different conditions. (**a**) GFP expression in hematopoietic progenitors treated with doxycycline for different lengths of time. Untreated hematopoietic progenitors (*red*) do not express GFP. Treatment with doxycycline for 2 days during differentiation is sufficient to induce GFP expression in hematopoietic progenitors (*blue*). When doxycycline was given for 8 days, the induction of GFP expression is stronger (*black*), suggesting that transgene expression does not get silenced when hESCs undergo hematopoietic differentiation. (**b**) The level of GFP expression in hematopoietic progenitors increases with the concentration of doxycycline added

also be adapted to accommodate the use of TALENs (8, 9) or (CRISPR)-Cas9 system (8, 10–12) in place of ZFNs.

As a proof of concept, we have generated a doxycycline-inducible human embryonic stem cell (hESC) line expressing GFP. This line was generated using an earlier version of this protocol, which utilized single drug selection. We have differentiated this line into hematopoietic progenitors or pancreatic beta cells and observed that GFP expression is visible 2 days after the addition of doxycycline and is maintained as long as doxycycline is present in the system. Importantly, the expression of GFP is proportional to the amount of doxycycline added and the length of time doxycycline is present in the medium (Fig. 3). This implies that we can induce a suitable, physiological level of transgene expression by

titrating different doses of doxycycline. Since then, we have developed an improved protocol described here, which uses dual drug selection to increase the specificity of selection and decrease the need to screen large numbers of clones.

2 Materials

2.1 Reagents and Supplies

2.1.1 Vectors Encoding Zinc Finger Nucleases Specific to the AAVS1 Locus

1. PGK-AAVS1-ZFN-Left (addgene cat. no. #60915).
2. PGK-AAVS1-ZFN-right (addgene cat. no. #60916).

2.1.2 Targeting Vectors To Be Integrated into the AAVS1 Locus

1. AAVS1-SA-2A-NEO-CAG-RTTA3 (addgene cat. no. #60431).
2. AAVS1-SA-2A-PURO-TRE-eGFP (addgene cat. no. #22074) (GFP can be replaced by any gene of interest).

2.1.3 Transfection Reagent

1. Lipid transfection reagent: X-tremeGENE 9 DNA transfection reagent (Roche Cat. no. 06 365 787 001).

2.1.4 Cell Culture Media and Reagents

1. Dulbecco's Modified Eagle's Medium/Ham's F12 50/50 mix (DMEM/F12 50/50) (Corning Cellgro Cat. no. 10-092-CV).
2. Knockout™ Serum Replacement (Knockout™ SR) (Life Technologies Cat. no. 10828-028).
3. Penicillin Streptomycin Solution (Pen-Strep), 100× (Corning Cellgro Cat. no. 30-002-Cl).
4. L-Glutamine Solution, 100× (Corning Cellgro Cat. no. 25-005-Cl).
5. MEM Nonessential Amino Acids (NEAA), 100× (Life Technologies Cat. no. 11140-050).
6. 2-Mercaptoethanol (55 mM) (Life Technologies Cat. no. 21985-023).
7. Iscove's Modification of DMEM (IMDM) (Corning Cellgro Cat. no. 10-016-CV).
8. PES Sterilizing 0.22 μm Filter System, Low Protein binding (250 ml) (Corning Cat. no. 431096).
9. Dimethyl sulfoxide (DMSO) (Sigma Cat. no. D2650).
10. Fetal Bovine Serum (Tissue Culture Biologicals Cat. no. 101).
11. TrypLE™ Express (1×), phenol red (Life Technologies Cat. no. 12605-010).

12. Trypsin-EDTA (0.25 %), phenol red (Life Technologies Cat. no. 25200-056).
13. Rock inhibitor Y-27632 dihydrochloride (TOCRIS Cat. no. 1254).
14. Basic Fibroblast Growth Factor (bFGF) (R&D Systems Cat no. 233-FB-025).
15. Matrigel Matrix, Growth Factor Reduced (Corning Cat. no. 354230).
16. Irradiated drug resistant (DR4) mouse embryonic fibroblasts (MEFs) (1.2×10^6/vial) (ATCC® SCRC-1045.1) (*see* **Note 1**).
17. Irradiated CF-1™ MEFs (1.2×10^6/vial) (ATCC® SCRC-1040.1).
18. Geneticin®/Neomycin (G418) (Life Technologies Cat. no. 10131035).
19. Puromycin dihydrochloride (Sigma Cat. no. P8833-25MG).

2.1.5 Molecular Biology Reagents

1. PureLink® Genomic DNA Mini Kit (Invitrogen Cat. no. K1820-01).
2. Platinum® Blue PCR SuperMix (Invitrogen Cat. no. 12580-015).
3. Screening primers reconstituted to 100 μM.
   (a) Primer set WT
      - WT-F: 5′ CCC CTA TGT CCA CTT CAG GA 3′
      - WT-R: 5′ CAG CTC AGG TTC TGG GAG AG 3′

   (b) Primer set AAVS1-CAG
      - AAVS1-CAG-F: 5′ GAG CAT CTG ACT TCT GGC TAA TA 3′
      - AAVS1-CAG-R: 5′ GAA GGA TGC AGG ACG AGA AA 3′

   (c) Primer set AAVS1-TRE
      - AAVS1-TRE-F: 5′ GCA ATA GCA TCA CAA ATT TCA C 3′
      - AAVS1-TRE-R: Same as AAVS1-CAG-R
4. Ultrapure Agarose (Invitrogen Cat. no. 16500-500).
5. 1 kb Plus DNA ladder (Invitrogen Cat. no. 10787-018).

2.1.6 Southern Blot Reagents

1. Probe: 480 bp fragment from the digestion of AAVS1-SA-2A-PURO plasmid (addgene cat. no. #22075) with BamHI restriction enzyme.

2. Restriction enzymes:
   (a) SphI-HF® (New England Biolabs Inc. Cat. no. R3182S).
   (b) BamHI (New England Biolabs Inc. Cat. no. R0136S).
3. Standard Southern blotting reagents.

2.2 Cell Culture Media

1. hESC medium—DMEM/F12 50/50, 1 % L-Glutamine, 1 % Pen-Strep, 1 % NEAA, 15 % Knockout™ SR, 100 μM 2-ME. Add reagents together in a PES Sterilizing 0.22 μm Filter System. Filter sterilize. Add bFGF to a final concentration of 5 ng/ml. Store at 4 °C for up to 10 days.
2. MEF medium—IMDM, 10 % serum, 1 % Pen-Strep, 1 % L-Glutamine. Add reagents together in a PES Sterilizing 0.22 μm Filter System. Filter sterilize. Store at 4 °C for up to a month.

3 Methods

Two days prior to transfection.

3.1 Preparation of 1:3 Matrigel/DR4 MEF Plates

- Thaw Matrigel on ice or overnight at 4 °C. Dilute 1:3 in cold IMDM in pre-cooled tubes on ice. Extra 1:3 Matrigel can be aliquoted and stored at −20 °C (*see* **Note 2**).
- Pre-cool a 6-well plate and 2 ml pipettes in the −20 °C freezer for 10 min.
- Coat the pre-cooled 6-well plate with diluted 1:3 Matrigel on ice using cold 2 ml pipettes. The whole surface should be completely covered with a thin coat of Matrigel.
- Incubate the plate on ice for 20 min.
- Aspirate excess 1:3 Matrigel from the wells.
- Incubate the 1:3 Matrigel-coated plate at 37 °C for at least 30 min, up to 4 h.
- Thaw one vial of irradiated DR4 MEFs (1.2×10^6/vial) in the 37 °C water bath.
- Add to 10 ml of IMDM in a 50 ml tube. Centrifuge 335 × *g* for 3 min. Aspirate and resuspend the cell pellet in 12 ml of MEF medium.
- Plate MEFs on the 1:3 Matrigel-coated 6-well plate at 2 ml/well.

One day prior to transfection.

3.2 Passaging iPSCs onto 1:3 Matrigel/DR4 MEFs for Transfection

- Aspirate hESC medium from wells of iPSCs to be split for transfection (*see* **Note 3**).

- Add 1 ml/well of TrypLE (warmed to room temperature), incubate for 3–4 min in the hood, and aspirate.
- Add 1 ml IMDM/well, incubate for 1 min, and repeat wash step.
- Add 1 ml/well hESC medium containing rock inhibitor (10 μM).
- Remove iPSCs using a cell scraper.
- Wash 1:3 Matrigel/DR4 MEF plates two times using 1 ml/well IMDM per well, and plate cells at a split ratio of 1:4 in hESC medium containing rock inhibitor (10 μM) (*see* **Note 4**).

Day of transfection.

3.3 Medium Change to Remove Rock Inhibitor

- Add 2 ml/well hESC media without rock inhibitor to cells first thing in the morning.

3.4 Transfection

- Add ZFN, TRE, and rtTA vectors to 1.5 ml Eppendorf tube bringing the total volume to 100 μl per tube using IMDM.
- Add XtremeGENE 9 DNA transfection reagent to IMDM/DNA mixture using 3 μl for every 1 μg of DNA.
- Mix the reagents by gently flicking the tube and incubate at room temperature for 20 min.
- Add the 100 μl of IMDM/DNA/lipid mixture to cells dropwise.
- Gently shake the plate to distribute the mixture uniformly in the well.
- Incubate for 16 h at 37 °C 5 % CO_2, 5 % O_2, and 90 % N_2.
- Aspirate transfection mixture and add 2 ml/well hESC medium.
- *See* **Notes 5** and **6** for transfection troubleshooting.

3.5 Selection of Transfected Colonies

- Start puromycin selection (0.5 μg/ml in hESC medium) 48 h after transfection. Feed cells with fresh hESC medium containing puromycin (2 ml/well) every day for 2 days.
- Feed cells with fresh hESC medium without drugs (2 ml/ well) for 2 days.
- Start neomycin selection (40 μg/ml in hESC medium). Feed cells with hESC medium containing neomycin (2 ml/well) every day for 8–10 days (*see* **Notes 7–9**)
- The day before picking clones, prepare 12-well plates containing 1:3 Matrigel/CF-1 MEFs.
- Pick colonies into 12-well plates containing 1:3 Matrigel/CF-1 MEFs and allow colonies to grow until 80–90 % confluent (*see* **Notes 10** and **11**).

- Passage the clones when 80–90 % confluent. Plate 25 % of the cells into 1 well of a 5-well plate and use the remaining 75 % for genomic DNA extraction.
- Expand and freeze individual clones (*see* **Note 12**).

3.6 PCR Screening of Double-Targeted Clones

Reagent	Amount/volume
Platinum® Blue PCR SuperMix	20 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Genomic DNA	100 ng (up to 5 µl)

- Perform PCRs using primer sets WT, AAVS1-CAG, and AAVS1-TRE listed in Sect. 2.1.5, item 3.
- PCR cycle:
  - Step 1—95 °C for 10 min
  - Step 2—95 °C for 30 s
  - Step 3—55 °C for 30 s
  - Step 4—72 °C for 2 min
  - Repeat steps 2–4 for 35 cycles
- Analyze PCR products on an ethidium bromide (EtBr) agarose gel for the following predicted band sizes:
  - WT (AAVS1 locus)—500 bp
    - Presence of a WT band indicates that the clone is heterozygous, thus not a double-targeted clone.
  - AAVS1-CAG-rtTA—1,920 bp
    - Presence of an AAVS1-CAG-rtTA band indicates CAG-RTTA plasmid integration at the AAVS1 locus
  - AAVS1-TRE—1,380 bp
    - Presence of an AAVS1-TRE band indicates TRE-GFP plasmid integration at the AAVS1 locus

3.7 Southern Blotting of Double-Targeted Clones to Reveal Off-Target Integrations

- Digest genomic DNA isolated from double-targeted clones with SphI-HF® restriction enzyme.
- Run digested DNA on a 0.7 % agarose gel.
- Transfer to membrane and probe using DNA fragment from AAVS1 vector backbone as described in Sect. 2.1.6.
- Southern blot results:
  - Nontargeted cells should result in a 6.5 kb fragment.
  - TRE target allele should result in a 3.4 kb fragment and CAG-rtTA in a 3.7 kb fragment.

3.8 Addition of Doxycycline to Induce Transgene Expression

As a proof-of-principle study, we generated a hESC line expressing doxycycline-inducible GFP. We differentiated the hESC line into hematopoietic progenitors, adding doxycycline (2 μg/ml) 2 days or 8 days before the emergence of the hematopoietic progenitors. The longer doxycycline was present in the media, the stronger the induction of GFP expression in the hematopoietic progenitors (Fig. 3a). The induction of GFP expression increases with the amount of doxycycline added (Fig. 3b). In addition, this proof-of-principle study establishes that the doxycycline system of inducing transgene expression does not get silenced through epigenetic mechanisms as iPSCs undergo hematopoietic differentiation, and thus is suitable for use in the study of blood disorders.

4 Notes

1. Irradiated drug resistant and normal MEFs are available commercially. Alternatively, MEFs can be expanded, irradiated, and frozen down at 1.2×10^6 cells/vial. Each vial is sufficient for one 6-well plate.
2. Keep tubes, plates, and pipette tips cold to prevent Matrigel from clumping. Frozen 1:3 Matrigel aliquots will thaw in a couple of hours or overnight at 4 °C.
3. iPSCs are ready to be split for transfection when they are 80–90 % confluent, with good morphology.
4. Passage cells the day before transfection and adjust split ratios to obtain small colonies that are 25–30 % confluent. Individual colonies should be small, as large colonies do not transfect well. Cells should be transfected the day after splitting as cells that were plated for longer than a day do not transfect well even if the confluency is right. We found a split ratio of 1:4 works best for our cell lines.
5. We recommend starting the transfection in the evening as prolonged incubation of cells with transfection reagent (>16 h) leads to less efficient transfection.
6. If you encounter the problem of one targeting vector integrating preferentially over the other, adjust the ratio of the two targeting vectors (*see* Table 2). If there are very few surviving clones after dual drug selection, we recommend transfecting more wells and/or increasing the amount of targeting vectors.
7. The doses for puromycin and neomycin work for several lines that we have tested. If there are problems with selection, we recommend doing a kill curve to titrate concentrations suitable for different iPSC lines.
8. We recommend a recovery period of 2 days after puromycin selection before starting neomycin selection. The colonies

Table 2
Recommended concentrations of reagents and vectors for transfection

Reagent/vector	Well #1	Well #2	Well #3
AAVS1-ZFN-Left	0.2 μg	0.2 μg	0.2 μg
AAVS1-ZFN-Right	0.2 μg	0.2 μg	0.2 μg
AAVS1-SA-2A-PURO-TRE-GFP	1.5 μg	1 μg	3 μg
AAVS1-SA-2A-NEO-CAG-RTTA	1.5 μg	3 μg	1 μg
IMDM	Top up to 100 μl total volume	Top up to 100 μl total volume	Top up to 100 μl total volume
XtremeGENE 9	10.2 μl	13.2 μl	13.2 μl
TRE-GFP:CAG-RTTA ratio	1:1	1:3	3:1
Total DNA	3.4 μg	4.4 μg	4.4 μg

should be very small when you begin neomycin selection as neomycin takes 8–10 days to kill. If colonies are too large or growing too fast, the colonies will overgrow before neomycin takes effect. Decrease recovery period to a day for fast growing cell lines.

9. Some DR4 MEFs may die during the whole process of drug selection. Supplement MEFs to the plate if necessary.
10. Clones are ready to be picked when the colony occupies half the view when viewed under the 10× magnification.
11. To increase the growth rate of hESC/iPSCs, increase the concentration of bFGF in the media up to 20 ng/ml.
12. Freeze down one 90 % confluent well of a 6-well plate. To freeze iPSCs, split cells with TrypLE as described above in Sect. 3.2. Instead of scraping cells into hESC media, scrape cells into pre-cooled freezing media: 50 % fetal bovine serum, 40 % hESC media, 10 % DMSO. Cells in freezing media can be immediately transferred to the −80 °C freezer. For long-term storage, keep frozen cells in liquid nitrogen.

Acknowledgments

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Generation and Characterization of Patient-Specific Induced Pluripotent Stem Cell for Disease Modeling

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Abstract

One major hurdle to the development of effective treatments to many diseases is the lack of suitable human model systems. The ability to reprogram human somatic cells to induced pluripotent stem cells (iPSC) offers an excellent opportunity to generate human disease models with primary cells. Currently, several methods to generate iPSC lines exist, and iPSC can be generated from various tissue sources including skin fibroblasts, blood, hair follicles, dental tissue, and urine. In this chapter we describe the generation and characterization of iPSC from blood or fibroblast on a routine base and focus on the integration-free methodologies.

Keywords: iPSC, Integration-free, Disease modeling, Parkinson's disease

1 Introduction

The discovery of induced pluripotent stem cells (iPSC) was first described in mice and then in human by Yamanaka and colleagues (1). iPSC has opened a new area in human stem cell research, personalized medicine, and transplantation therapies (2–5). In Yamanaka's seminal work, four transcription factors, Oct3/4, Sox2, c-Myc, and Klf4, were delivered to fibroblasts by retroviral vectors to reprogram to iPSC. The technology has since been modified by fewer transcription factors or small molecules, and delivering by nonviral methods (6–9).

Our laboratory has focused on integration-free reprogramming methods, as any complications that may associate with the reactivation of reprogramming factors are expected to be minimal by a non-integrating method. We routinely used two stable systems for reprogramming of different somatic cell types: (1) Sendai virus reprogramming (10) and (2) replicative episomal vector reprogramming (11). The former is a negative-strand RNA virus that does not integrate into the host genome. This virus replicates in the host cytoplasm and is nonpathogenic to humans. Recent studies have shown high efficiencies in reprogramming of a variety of somatic cell types using Sendai virus (10, 12–14). The latter is a

plasmid that contains two elements from Epstein-Barr (EB) virus, the origin of viral replication (oriP) and the EB nuclear antigen 1 (EBNA). oriP facilitates replication of the plasmid in human cells, and EBNA mediates replication and dividing simultaneously with host division. The episomal vectors in general diminish with each cell division and are totally depleted after approximately 2 months in culture (11, 15).

In this chapter we specifically describe the reprogramming of fibroblasts obtained from Parkinson's disease (PD) patients by Sendai and $CD34^+$ blood cord cells by episomal vectors. We also describe the routine characterization of generated iPSC including expression of pluripotency markers, ability to differentiate in vitro, genome stability in prolonged culture, and validation of non-integration. Such iPSC lines are suitable for developing clinical applications in the future or for drug screening.

2 Materials

All cells are maintained in an incubator with 5 % CO_2 at 37 °C. Mediums are prepared in tissue culture hood using aseptic techniques.

2.1 Tissue Culture

2.1.1 Supplies

- Antibiotic-antimycotic 100× (Anti/Anti) (Life Technologies; New York, NY; cat. no. 15240062).
- Collagenase, Type IV (Life Technologies; cat. no. 14104-019).
- CytoTune®-iPS Sendai Reprogramming Kit (SeV) (Life Technologies; A1378001).
- CryoStem Freezing Medium (Stemgent, Cambridge, MA; cat. no. 01-0013-50).
- Dulbecco's Modified Eagle's Medium/Ham's (DMEM) high glucose, pyruvate (Life Technologies; cat. no. 11995-040).
- Deoxyribonuclease I (DNase I) (Sigma, St. Louis, MO; cat. no. D4513).
- Dulbecco's Phosphate-Buffered Saline, 1× (PBS) (Corning cellgro, Manassas, VA; cat. no. 21-030-CV).
- EmbryoMax® 0.1 % Gelatin Solution (EMD Milipore, Billerica, MA; cat. no. ES-006-B).
- Fetal Bovine Serum (FBS) (Life Technologies; cat. no. 16000044).
- Geltrex LDEV Free (Life Technologies; cat. no. A11343-01).
- Glasgow's MEM (GMEM) (Life Technologies; cat. no. 11710035).

- GlutaMAX™-I (100×) (Life Technologies; cat. no. 35050061).
- Hematopoietic™ Progenitor Growth Medium (HPGM™) (Lonza, Walkersville, MD; cat. no. PT-3926).
- HumanES (hES) Cloning and Recovery Supplement (Stemgent; cat. no. 01-0014-500).
- Knockout™ DMEM/F12 (Life Technologies; cat. no. 12660012).
- Knockout™ Serum Replacement (Life Technologies; cat. no. 10828028).
- Minimum Essential Medium (MEM) Non-essential Amino Acids 100× (Life Technologies; cat. no. 11140-050).
- Minimum essential medium alpha (MEM-alpha), nucleosides, GlutaMAX™ (Life Technologies; cat. no. 32571-101).
- Mitomycin-C from *Streptomyces caespitosus* (Sigma; cat. no. M0503).
- Mr. Frosty Freezing Container (Thermo Fischer Scientific, Waltham, MA; cat. no. 5100-001).
- Nucleofector Kit ($CD34^+$ cells) (Lonza; cat. no. VPA-1003).
- Plasmid EBNA1-C5 vector carrying OCT4, SOX2, KLF4, LIN28, c-MYC reprogramming factors (Addgene, Cambridge, MA; Plasmid 28213 http://www.addgene.org/28213/).
- Plasmid EBNA1-Tg vector carrying SV40 large T antigen (Addgene; Plasmid 28220 http://www.addgene.org/28220/).
- Recombinant Human Flt3-Ligand (FL) (PeproTech, Rocky Hill, NJ cat. no. AF-300-19).
- Recombinant Human Stem Cell Factor (SCF) (PeproTech; cat. no. AF-300-07).
- Recombinant Human Thrombopoietin (TPO) (PeproTech; AF-300-18).
- ROCK inhibitor Y27632 (Stemgent; cat. no. 04-0012).
- Stemfactor Fibroblast Growth Factor-basic (Human Recombinant) (bFGF) (Stemgent; cat. no. 03-0002).
- Sodium Butyrate (Na Butyrate) (Stemgent; 04-0005).
- Sodium Pyruvate (Life Technologies; cat. no. 11360070).
- TWEEN 20 (Sigma; cat. no. P5927).
- 2-Mercaptoethanol 55 mM (Life Technologies; cat. no. 21985023).
- 60 mm Not TC-Treated Culture Dish (Corning; cat. no. 430589).

2.1.2 Medium

1. Basic FGF solution:

100 μg/mL basic FGF solution
Basic FGF 50 μg
0.1 % BSA solution in 1× PBS 500 μL

Make aliquot and store at −20 °C up to 6 months. Thaw an aliquot and keep at 2–8 °C up to 1 week.

2. 1 mg/mL Collagenase IV solution:

50 mL Collagenase IV solution
Collagenase IV 50 mg
Knockout™ DMEM/F12 50 mL

Dissolve Collagenase IV in Knockout™ DMEM/F12 and filter. Make aliquot and store −20 °C up to 6 months. Thaw an aliquot and keep at 2–8 °C up to 1 week.

3. Medium for Mouse Embryonic Fibroblast (MEF) cells:

100 mL MEF medium	Final concentration
DMEM, high glucose, pyruvate 89.4 mL	
FBS 10 mL	10 %
Antibiotic-Antimycotic 100× 600 μL	0.6 %

Filter medium and store at 2–8 °C up to 1 week.

4. Medium for hES/iPSCS cells:

100 mL hES/iPSC medium	Final concentration
Knockout™ DMEM/F12 75 mL	
Knockout™ Serum Replacement 20 mL	20 %
GlutaMAX™-I 100× 1 mL	1 %
Non-essential amino acids 100× 1 mL	1 %
Antibiotic-Antimycotic 100× 1 mL	1 %
2-Mercaptoethanol 55 mM 186 μL	0.1 mM
Basic FGF 100 μg/mL 4 μL	4 ng/mL

Filter medium without bFGF. Add bFGF after filtration and store at 2–8 °C up to 1 week.

5. Medium for EB:

100 mL EB medium	Final concentration
Knockout™ DMEM/F12 75 mL	
Knockout™ Serum Replacement 20 mL	20 %
GlutaMAX™-I 100× 1 mL	1 %
Non-essential amino acids 100× 1 mL	1 %
Antibiotic-antimycotic 100× 1 mL	1 %
2-Mercaptoethanol 55 mM 186 μL	0.1 mM

Filter medium and store medium at 2–8 °C up to 1 week.

6. Medium for $CD34^+$ cells:

100 mL CD 34^+ medium	Final concentration
HPGM™ 100 mL	
Recombinant Human FL (100 ng/μL)* 50 μL	50 ng/mL
Recombinant Human TPO (100 ng/μL)* 50 μL	50 ng/mL
Recombinant Human SCF (100 ng/μL)* 25 μL	25 ng/mL

*Recombination factors were diluted in 5 % trehalose. Aliquot the recombination factors and store in −20 °C for up to 6 months. Filter medium without recombination factors. Add factors after filtration and store at 2–8 °C up to 1 week.

7. Parkinson's disease patient-specific fibroblast medium (PD medium):

100 mL PD medium	Final concentration
MEM-alpha, nucleosides, GlutaMAX™ 83 mL	
FBS 15 mL	15 %
Non-essential amino acids 100× 1 mL	1 %
Antibiotic-antimycotic 100× 1 mL	1 %

Filter medium and store medium at 2–8 °C up to 1 week.

8. MEF-Condition Medium (MEF-CM):
   (a) Thaw a vial of untreated MEF cells in 37 °C water bath.

(b) Transfer MEF to a 15 mL centrifuge tube containing 5 mL pre-warmed MEF medium and centrifuge for 3 min at 2500 × *g*.

(c) Aspirate medium and resuspend pellet in 550 mL MEF medium.

(d) Slowly add diluted MEF cells in a HYPERflask.

(e) When adding or poring medium to HYPERflask, tilt it to one side to avoid air bubbles. Tap on the side of the flask to remove air bubbles. Apply aspirator to remove excess air bubbles at the neck of the HYPERflask.

(f) Leave the MEF cells in HYPERflask for 24 h to settle and incubate at 5 % CO_2 at 37 °C.

(g) After 24 h change medium to 550 mL hES/iPSC medium.

(h) Change and collect hES/iPSC medium for 10 days. Collected medium from Day 1 should be discarded as it contains trace of MEF medium and dead MEF cells.

(i) Collected MEF-CM can be used immediately or store in −20 °C for up to 6 months.

(j) Always filter MEF-CM before use and add desired amount of bFGF.

(k) Avoid repeated thaw/freeze of MEF-CM medium.

2.1.3 Cells

- $CD34^+$ cells (Lonza; cat. no. 2C-101).
- Mouse Embryonic Fibroblast (MEF), Mitomycin-C treated (GlobalStem, Rockville, MD; cat. no. GSC-6001M).
- MEF untreated (GlobalStem; cat. no. GSC-6001).
- Parkinson's disease fibroblasts (Coriell Institute, Camden, New Jersey).
- WA09 (MEF Platform), H9, Human Embryonic Stem cell (16) (WiCell Research Institute, Madison, WI).

2.2 Immunocytochemistry

2.2.1 Primary Antibodies

- Mouse Anti-α-Fetoprotein (AFP), immunoglobulin (Ig)G (Sigma; cat. no. A8452).
- Mouse Anti-β-Tubulin III (Tuj), IgG (Sigma; cat. no. T8660).
- Mouse Anti-Actin, α-Smooth Muscle (17), IgG (Sigma; cat. no. A2547).
- Mouse Anti-NANOG, IgG (eBioscience, San Diego, CA; cat. no. 14-5768-82).
- Mouse Anti-SOX2, IgG (EMD Milipore; cat. no. MAB4343).
- Mouse Anti-TRA-1-60, IgM (eBioscience; cat. no. 14-8863-82).
- Rabbit Anti-OCT4A IgG (Cell Signaling Technology, Inc, Danvers, MA; cat. no. C52G3).

2.2.2 Secondary Antibodies

- Alexa Fluor 488 Goat Anti-Mouse IgG (Life Technologies; cat. no. A11029).
- Alexa Fluor 488 Goat Anti-Mouse IgM (Life Technologies; cat. no. A21042).
- Alexa Fluor 594 Goat Anti-Mouse IgG (Life Technologies; cat. no. A11032).
- Alexa Fluor 568 Goat Anti-Rabbit IgG (Life Technologies; cat. no. A11036).

2.2.3 Other Reagents

- Blood & Cell Culture DNA Mini Kit (QIAGEN, Valencia, CA; cat. no. 13323).
- GoTaq Green Master Mix (Promega, Madison, WI; cat. no. M7122).
- RNeasy Plus Mini Kit (QIAGEN; cat. no. 74134).
- iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA; cat. no. 170-8841).
- iTaq Universal SYBR Green Supermix (Bio-Rad; cat. no. 172-5124)
- Primers (**see** Table 1).
- Alkaline Phosphatase Staining Kit II (16) (Stemgent; cat. no. 00-0055).

Table 1
Primer sequences

Target	Primer sequence	Amplicon bp/annealing °C
PCR		
EBNA 1	*5′-TTTAATACGATTGAGGGCGTCT-3′* *5′-GGTTTTGAAGGATGCGATTAAG-3′*	244 bp/51.2 °C
TG	*5′-GCCAGGTGGGTTAAAGGAGC-3′* *5′-GGTACTTATAGTGGCTGGGCTGT-3′*	200 bp/54.4 °C
SK	*5′-CCATTAACGGCACACTGCCCCTGT-3′* *5′-AGGACGGGAGCAGAGCGTCGCTGA-3′*	135 bp/62.2 °C
qPCR		
Nanog	*5′-TGAACCTCAGCTACAAACAG-3′* *5′-TGGTGGTAGGAAGAGTAAAG-3′*	1,493 bp/60 °C
OCT 4 ENDO	*5′-CCTCACTTCACTGCACTGTA-3′* *5′-CAGGTTTTCTTTCCCTAGCT-3′*	124 bp/60 °C
OCT 4 TOTAL	*5′-AGCGAACCAGTATCGAGAAC-3′* *5′-TTACAGAACCACACTCGGAC-3′*	362 bp/60 °C
SOX2 ENDO	*5′-CCCAGCAGACTTCACATGT-3′* *5′-CCTCCCATTTCCCTCGTTTT-3′*	112 bp/60 °C
SOX2 TOTAL	*5′-AGCTACAGCATGATGCAGGA-3′* *5′-GGTCATGGAGTTGTACTGCA-3′*	86 bp/60 °C

(continued)

Table 1
(continued)

Target	Primer sequence	Amplicon bp/annealing °C
B-Actin	*5′-TGAAGTGTGACGTGGACATC-3′* *5′-GGAGGAGCAATGATCTTGAT-3′*	224 bp/60 °C
SeV	*5′-GGATCACTAGGTGATATC GAGC-3′* *5′-ACCAGACAAGAGTTTAAGAGATATGTATC-3′*	181 bp/60 °C

Fixatives

- Paraformaldehyde, 16 % w/v aq. soln., methanol free (Alfa Aesar, Ward Hill, MA; cat. no. 43368).

Blocking buffer

- Bovine Serum Albumin 25 % (BSA) (Life Technologies; cat. no. A10008-01).
- Goat Serum (Sigma; cat. no. G9023).
- Triton X-100, 10 % solution, peroxide-free (Amresco, Solon, OH; cat. no. M236).
- Dulbecco's Phosphate-Buffered Saline, 10× (Corning; cat. no. 20-030-CV).

Antifade/Hoechst mixture

- Hoechst 33342, Trihydrochloride, Trihydrate (Life Technologies; cat. no. H3570).
- ProLong Gold Antifade Reagent (Life Technologies; cat. no. P366934).

10 mL of 2 % fixative		Final concentration
Paraformaldehyde, 16 % w/v aq. soln., methanol free	1.25 mL	2 %
Miliq H_2O	8.75 mL	

Always prepare fresh 2 % Paraformaldehyde for fixation of cells.

1 mL of blocking buffer		Final concentration
Bovine Serum Albumin (BSA), 10 % working solution	100 µL	10 %
Goat Serum	100 µL	10 %
Triton X-100, 10 % solution, peroxide-free	30 µL	0.3 %
Dulbecco's Phosphate-Buffered Saline (PBS) 10×	100 µL	1×
Miliq H_2O	670 µL	

Always prepare fresh blocking buffer. Dilute primary and secondary antibody in blocking buffer.

Antifade/Hoechst	
Hoechst 33342, Trihydrochloride, Trihydrate	1 µL
ProLong Gold Antifade Reagent	5,999 µL

Prepare Hoechst 33342 in ProLong Gold in 1:6,000. Make aliquot and store them in −20 °C for up to 1 year. Keep a thawed aliquot in 2–8 °C for up to 2 weeks.

Alkaline Phosphatase Staining Kit II		Final concentration
10 mL PBS TWEEN (PBST)		
TWEEN 20	5 µL	0.05 %
Dulbecco's Phosphate-Buffered Saline, 1× PBS	10 mL	

Keep 0.05 % PBST at RT for up to 1 month.
AP Substrate solution from Alkaline Phosphatase Staining Kit II

1. Mix solution A and B (1:1) and leave at RT for 2 min.
2. Add solution C in equal amount as A. Total solution mixture is (1:1:1).
3. AP Substrate solution should be used within 30 min, after preparation. Always make fresh AP solution.

3 Methods

3.1 Coating Plates

3.1.1 Gelatin-Coated Plates

Incubate d35mm or d60mm tissue culture plates with 0.1 % Gelatin Solution for 1 hour (h) at room temperature (RT) under sterile conditions.

3.1.2 Geltrex-Coated Plates

1. Thaw Geltrex on ice. Make aliquot and store at −20 °C up to 6 months.
2. Dilute Geltrex 1:50 in Knockout™ DMEM/F12.
3. Cover dish completely with Geltrex mixture at RT for 1 h.
4. Use plates immediately or store in 4 °C for up to 2 weeks.

3.2 Preparation of MEF Feeder Layer

1. Thaw a vial of MEF cells in a 37 °C water bath.
2. Transfer MEF to a 15 mL centrifuge tube containing 5 mL pre-warmed MEF medium and centrifuge for 3 min at 2500 × *g*.
3. Aspirate medium and resuspend pellet in 5 mL MEF medium and plate 5×10^5 cells on a pre-coated d60mm dish with gelatin. Incubate cells at 37 °C, 5 % CO_2 overnight (O/N). After 24 h, the cell should have formed a confluent monolayer. When plating hES/iPSC change medium accordingly from MEF medium to hES medium or MEF-CM + bFGF.

3.3 Thaw Fibroblast

1. Thaw a vial of PD fibroblast cells in a 37 °C water bath.
2. Transfer fibroblast to a 15 mL centrifuge tube containing 5 mL pre-warmed PD medium and centrifuge for 3 min at 2500 × *g*.
3. Aspirate medium and add 15 mL PD medium.
4. Transfer PD cells in a T75 flask and grown until confluence.

3.4 Episomal Vector Reprogramming of $CD34^+$ Cells (Fig. 1a)

1. Day 1:
   (a) Apply Amaxa program U-008 to nucleoporate 1×10^6 $hCD34^+$ with 8 μg of C5 plasmid and 2 μg of Tg.
   (b) Transfer nucleoporated cells to 1 well in a 12-well plate with $CD34^+$ medium enhanced with cytokines for 2 days.
2. Day 3:
   (a) Transfer nucleoporated $CD34^+$ cells to a 3-well coated with MEF cells in a 12-well plate, in MEF medium.
   (b) Spin down plate for 100 × *g* for 30 min to assist nucleoporated cells attachment to MEF-coated wells. Cells will be maintained in MEF medium for 1 day.
3. Day 4:
   (a) Replace MEF medium with hES/iPSC medium supplemented 10 ng/mL bFGF (**Note 1**). Add Na-butyrate to hES/iPSC medium. Final concentration of Na-butyrate 0.25 mM.
   (b) Change hES/iPSC medium supplemented with 10 ng/mL bFGF and Na-butyrate 0.25 mM every other day for total of 6 days.
4. Day 10:
   (a) Switch medium to MEF-CM with 10 ng/mL bFGF.
5. Day 14:
   (a) Perform TRA-1-60 staining on live colonies/cells, to identify possible iPSC clones (**Note 2**).
   (b) Manually dissect single TRA-1-60-positive clones to individual wells in a MEF-coated 12-well plate. An individual colony becomes a clone. For improve attachment and

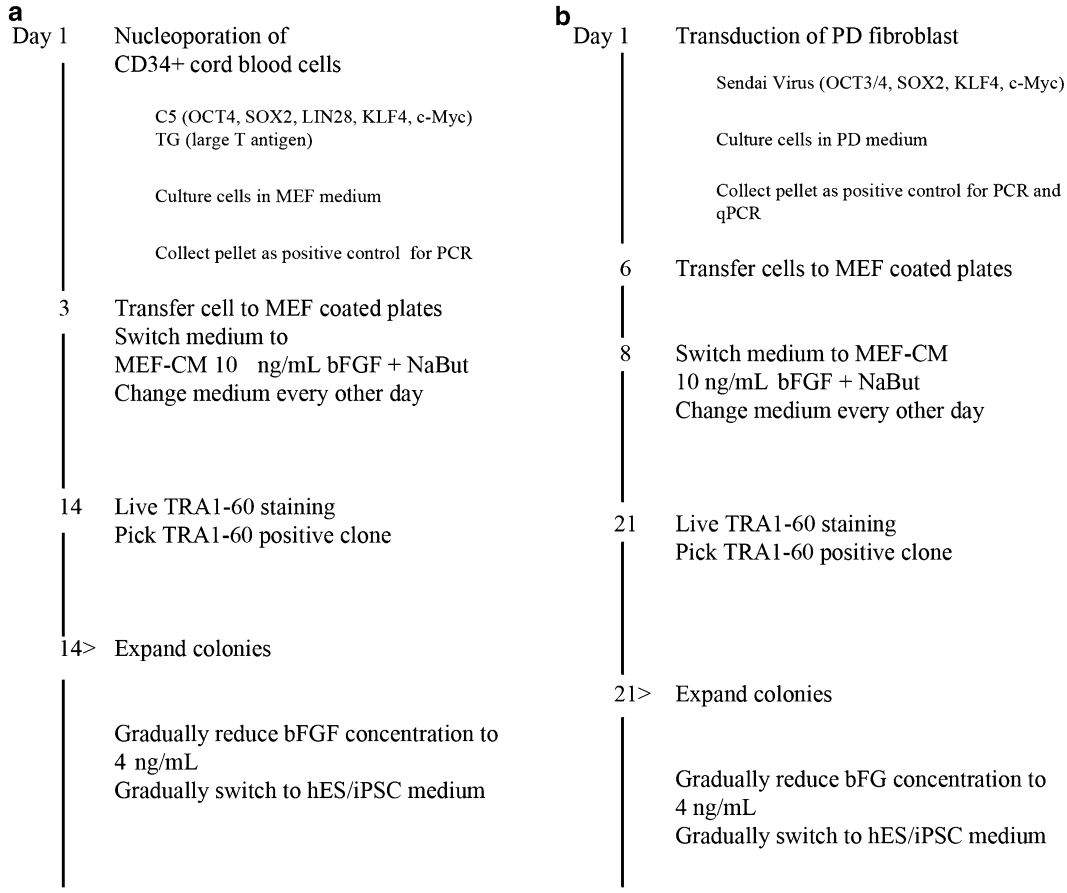


Fig. 1 Reprogramming of human somatic cells by integration-free methodologies. A schematics and timeline of the reprogramming process is illustrated for the episomal vector method (**a**) and Sendai virus method (**b**)

survival of colony in each well, add 10 μM ROCK inhibitor and hES Cloning and Recovery Supplement.

6. Maintain the clones in 12-well plates for the first 2–3 passages and then expand them to d35mm dishes, followed by d60mm dish.
7. Manually passage clones until the first 6–10 passage. If less 10 % differentiation occurs, then switch to 1 mg/mL collagenase. Continue to use enzymatic passage if less than 10 % differentiation occurs after first collagenase treatment.
8. Continue manual passaging if more than 10 % differentiation occurs.
9. When clones are stable and undifferentiating, gradually decrease the bFGF level to 4 ng/mL in MEF-CM.
10. Gradually switch to 4 ng/mL bFGF hES/iPSC medium by mixing MEF-CM and hES/iPSC medium (**Note 3**).

3.5 Sendai Virus (SeV) Reprogramming of PD Fibroblast (Fig. 1b)

1. Thaw and grow PD fibroblast until confluent.
2. Day 1:
   (a) Plate 500 K PD cells on a d35mm dish in PD medium.
3. Day 2:
   (a) Thaw SeVs by immersing them in 37 °C water bath. Infect PD fibroblast with SeVs in fibroblast medium. Add volume of each virus accordingly to manufacturer recommendation. Incubate the cells at 37 °C, 5 % CO_2 O/N.
4. Day 3:
   (a) Remove virus-containing medium after 24 h and wash three times with PBS and add fresh fibroblast medium. No cell death should be observed at this stage.
5. Feed fibroblast every other day with fresh fibroblast medium. Observe changes in morphology and cell death.
6. Day 6–8:
   (a) Add trypsin to plate and incubate for 3 min at 37 °C, 5 % CO_2 or until cells detach.
   (b) Transfer cells to 15 mL conical tube and spin down for 3 min at 1500 × *g*.
   (c) Resuspend cells in fibroblast medium and count cells. Plate 150 K cells per d60mm coated with MEF cells.
   (d) Set aside remaining cells for RNA extraction, to be used as a positive control when detecting for presence of SeV genome in iPSC.
7. Day 8–10: Remove fibroblast medium and add MEF-CM supplemented with 10 ng/mL bFGF and 0.25 mM NA-butyrate.
8. Change medium every other day for 2–3 weeks and monitor cells.
9. 3 weeks after SeV transduction:
   (a) Perform TRA-1-60 staining on live cells, to identify possible positive iPSC clones.
   (b) Pick positive TRA-1-60 clones and transfer each colony to an individual well in a MEF-coated 12-well plate. Add ROCK inhibitor and hES Cloning and Recovery Supplement for enhanced attachment and survival of single colonies.
10. Maintain the clones in 12-well plates for the first 2–3 passages and then expand them to d35mm dishes, followed by d60mm dish.
11. Manually passage clones until the first 6–10 passage. If less 10 % differentiation occurs, then switch to 1 mg/mL collagenase. Continue to use enzymatic passage if less than 10 % differentiation occurs after first collagenase treatment.

12. Continue manual passaging if more than 10 % differentiation occurs.
13. When clones are stable and undifferentiating, gradually decrease the bFGF level to 4 ng/mL in MEF-CM.
14. Gradually switch to 4 ng/mL bFGF hES/iPSC medium by mixing MEF-CM and hES/iPSC medium (**Note 3**).

3.6 Freezing iPSC

1. Add collagenase to iPSC and incubate for 1 h at 37 °C, 5 % CO_2.
2. Collect medium with detached colonies in 15 mL conical tube and centrifuge at 1500 × *g* for 2 min.
3. Aspirate medium.
4. Add 500 μL CryoStem Freezing Medium and gently resuspend.
5. Transfer medium with colonies to a freezing vial and store in Mr. Frosty Freezing container at −80 °C for 24 h.
6. After 24 h move vial to a liquid nitrogen freezer.

3.7 Thaw iPSC

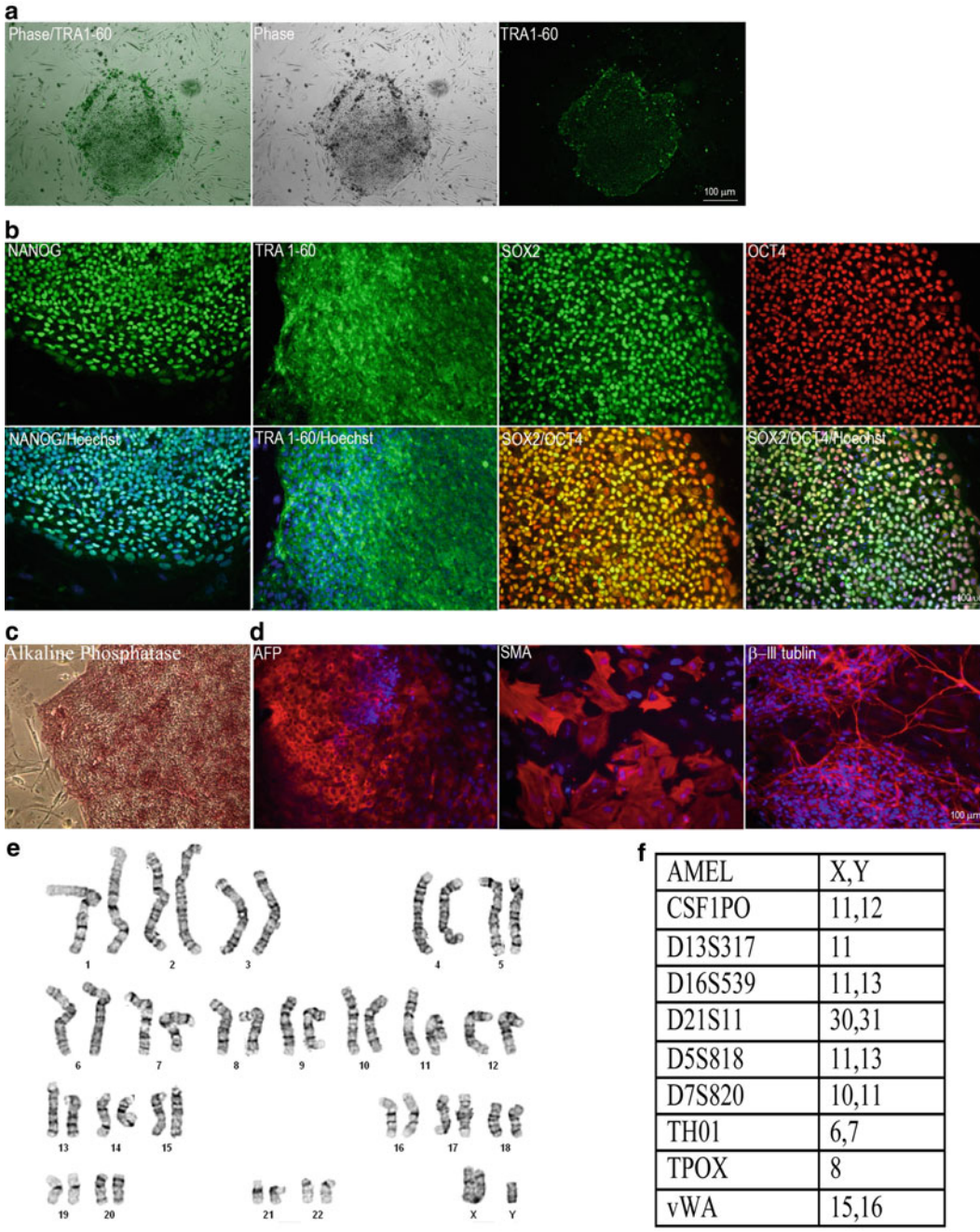
1. Thaw a vial of iPSCs in a 37 °C water bath.
2. Transfer fibroblast to a 15 mL centrifuge tube containing 5 mL pre-warmed hES/iPSC medium + 10 ng/mL bFGF and centrifuge for 2 min at 1,200 rpm.
3. Aspirate medium and add 1 mL hES/iPSC medium + 10 ng/mL bFGF.
4. Gently resuspend iPSC in hES/iPSC medium + 10 ng/mL bFGF without breaking them into single cells.
5. Transfer iPSCs in an MEF-covered petri dish and incubate at 37 °C, 5 % CO_2, for 48 h.
6. Observe and change hES/iPSC medium + 10 ng/mL bFGF every day after 48 h.
7. When possible passage colonies and when stable, slowly decrease bFGF concentration to 4 ng/mL, maintain cells in hES/iPSC medium (**Note 3**).

3.8 Basic Characterization

Basic characterizations of all new derived iPSC lines are required regardless of applied reprogramming method (Fig. 2). Newly derived iPSC lines should maintain pluripotency through own genome at passage 10>; hence, basic characterizations are performed at passage 10 or above.

3.8.1 TRA-1-60 Live Staining (Fig. 2a)

1. Add fresh medium to plate with primary antibody (1:60 dilution).
2. Incubate at 37 °C, 5 % CO_2 incubator for 15–30 min.



f

AMEL	X,Y
CSF1PO	11,12
D13S317	11
D16S539	11,13
D21S11	30,31
D5S818	11,13
D7S820	10,11
TH01	6,7
TPOX	8
vWA	15,16

Fig. 2 Characterization of iPSC lines generated by integration-free methods. Live label with TRA-1-60 antibody of ESC-like colonies appeared 10 days after reprogramming (**a**). Expression of pluripotency markers, NANOG, TRA1-60, SOX2 and OCT-4A (**b**, **c**). Differentiation into cells of the three germ layers (**d**). The iPSC remained a normal karyotype after 10 passages in vitro (**e**), and (**f**) STR analysis confirmed the identification of the cells at passage 10

3. Remove medium with antibody and rinse with fresh medium or PBS.
4. Add secondary antibody in fresh medium (Goat Anti-Mouse IgM, 1:1,000 dilution).
5. Incubate at 37 °C, CO_2 incubator for 15–30 min.
6. Remove medium with secondary antibody, rinse and add fresh medium.
7. Image cells right away (**Note 4**).

3.8.2 Immunocytochemistry (Fig. 2b)

To verify pluripotency of selected clones, perform staining for pluripotency markers, apply NANOG, TRA1-60, OCT-4A, and SOX2 antibodies:

1. Aspirate medium from dish.
2. Add 2 % Paraformaldehyde (PFA) and incubate for 20 min at RT.
3. Wash three times with 1× PBS.
4. Add blocking buffer for 1 h.
5. Aspirate and add blocking buffer containing primary antibody.
6. Incubate O/N at 4 °C.
7. Wash three times with 1× PBS.
8. Add blocking buffer containing secondary antibody. Incubate at RT for 1 h. Shield from light.
9. Wash three times with 1× PBS.
10. Add Antifade/Hoechst mixture.
11. Cover with cover slip.
12. Image cells or store in dark at RT for up to 24 h.

3.8.3 Alkaline Phosphatase (Fig. 2c)

Alkaline phosphatase is expressed by pluripotent stem cells and is used as another pluripotency marker:

1. Wash cells with 1× PBS.
2. Fix cells using fix solution provided in AP Kit and store at RT for 3 min.
3. Aspirate fix solution and wash with 0.05 % PBST.
4. Aspirate 0.05 % PBST and add prepared AP Substrate solution.
5. Store cells in dark for 5–15 min at RT. Longer exposure will demonstrate a darker AP staining and possibility of nonspecific staining.
6. Aspirate AP Substrate solution and wash twice with 1× PBS.
7. Cover cells with 1× PBS.
8. Image cells or store plate at 4 °C in dark for up to 48 h.

3.8.4 Spontaneous Differentiation (Fig. 2d)

One requirement of embryonic stem cells is to differentiate into all human cell lineages. To test this, we stain for all three germ layers using specific endoderm, mesoderm, and ectoderm markers. Apply AFP for endoderm, SMA for mesoderm, and Tuj marker for ectoderm:

1. Add 1 mg/mL collagenase to iPSC and incubate 1 h at 37 °C, 5 % CO_2.
2. Transfer cells to 15 mL conical tube and let them settle.
3. Aspirate collagenase.
4. Resuspend cells in hES/iPSC medium + 4 ng/mL bFGF. Be gentle when resuspending (**Note 5**).
5. Transfer cells to an ultralow attachment dish and add ROCK inhibitor (**Note 6**).
6. Place the plate in 37 °C, 5 % CO_2 for 24 h.
7. After 24 h collect cells in a 15 mL conical tube and let them settle.
8. Aspirate medium and switch medium to EB medium (**Note 7**).
9. Change medium every other day for 2 weeks.
10. After 2 weeks, attach EBs to a Geltrex-coated dish in EB medium.
11. Change medium every other day for another 10 days.
12. Proceed with staining cells for three germ layers.

3.8.5 Karyotype (Fig. 2e)

Reprogramming can induce karyotypic abnormal cell lines. Test all derived iPSC lines for normal karyotype. Routinely test iPSC lines for karyotype, approximately every 6 months. External resources performed our karyotype testing.

3.8.6 Short Tandem Repeat (STR) Analysis (Fig. 2f)

To verify that derived iPSC line originates from fibroblast, an STR analysis should be performed for both fibroblast and iPSC. STR analysis kits are available; however, we have external resources performing this test for us. Figure 2f illustrates applied STR markers:

- Add trypsin or collagenase to plate and incubate at 37 °C, 5 % CO_2 until cells detach.
- Transfer cells to 15 mL conical tube and spin down for 3 min at 1500 × *g*.
- Aspirate medium.
- Extract DNA using DNA Extraction protocol provided by QIAGEN (cat. no. 13323).

3.8.7 iPSC Bank and Recovery

One hurdle with iPSCs is the low recovery after freezing. After all basic characterization, make a bank of 20 vials with same passage from all newly derived iPSC lines and test recovery of each line. Twenty–thirty percent confluency after 14–21 days is regarded as a successful recovery.

3.9 Specific Characterization

Each reprogramming method requires specific experiments to verify no trace of applied delivery method and to ensure pluripotency is maintained through the newly derived iPSC line's genome.

3.9.1 RT-PCR (RNA Extraction, Reverse Transcription, PCR)

3.9.2 DNA or RNA Extraction

Specific characterization for episomal or Sendai reprogramming method require either DNA or RNA.

1. Add either collagenase or trypsin-EDTA to cells and incubate at 37 °C, 5 % CO_2 until cells detach.
2. Collect cells in a 15 mL conical and centrifuge for 2 min at 1500 × *g*.
3. Aspirate medium and follow RNA Extraction protocol provided by QIAGEN (cat. no. 74134) or DNA Extraction protocol provided by QIAGEN (cat. no. 13323).

3.9.3 Reverse Transcription

1. Mix 2 μg RNA, 4 μL Superscript II Reverse transcriptase, add up to 20 μL H_2O.
2. Vortex and spin down mixture.
3. Place tubes in a thermal cycler and use following protocol:

Priming	5 min at 25 °C
Reverse transcription	30 min at 42 °C
RT inactivation	5 min at 85 °C
Hold	4 °C

3.9.4 qPCR

Characterization Specific for Sendai Virus Reprogramming Method

To verify that iPSC express pluripotency genes from own genome and not by Sendai virus, perform qPCR for endogenous expression of pluripotency genes (OCT-4A and SOX2) and compare to total expression. Use NANOG as positive control gene, since it is not used in reprogramming cocktail. Also, test for presence of Sendai virus in derived iPSC lines. Use fibroblast as negative control:

1. Prepare qPCR mixture: 8 μL cDNA, 1 μL (10 μM) Forward primer, 1 μL (10 μM) Reverse primer, 10 μL 2× iTaq Universal SYBR Green Supermix.
2. Place tubes in thermal and fluorescence cycler and run desired cycle.
3. Data analysis using ΔCt method.

Apply Nanog, Oct4, SOX2, SeV, and B-Actin primers (Table 1).

3.9.5 PCR

Characterization Specific for Episomal Vector Reprogramming Method

To verify no presence of episomal vector, perform PCR on all derived iPSC lines on genes from both C5 and Tg vectors. Use fibroblast as negative control, 2 days post transfected fibroblast and vectors as positive control:

1. Test presence of reprogramming plasmid in reprogrammed iPSC cell lines.
2. Extract DNA from iPSC with passage 10 or higher (**Note 8**).
3. Extract DNA from $CD34^+$ cells 2 days after nucleoporation (positive control) and non-nucleoporated $CD34^+$ cells (negative control). Apply plasmid C5 and Tg as plasmid controls.
4. Prepare PCR mixture:

   100 ng DNA

   1 μL (1 μM) Forward + Reverse primer mixture

   10 μL 2× GoTaq Green Master

   H_2O up to 20 μL.

   Apply EBNA 1, SK, and TG primers. (Table 1).
5. Place tubes in thermal cycler and run cycle with annealing temperature as in Table 1.

4 Notes

1. Some $CD34^+$ cells do not attach first day, for better attachment collect MEF medium and spin it down at 100 × *g* for 5 min. Aspirate medium and resuspend cell pellet in hES/iPSC medium supplemented with 10 ng/mL bFGF.
2. Expect appearance of colonies with cord blood $CD34^+$ around 7–11 days post-nucleoporation. Expect appearance of colonies with adult bone marrow and peripheral blood $CD34^+$ cells around 11–14 days post-nucleoporation.
3. Observe colonies when decreasing bFGF levels and switching to hES/iPSC medium. If colonies differentiate more than 10 %, maintain colonies in existing medium for a little longer and then slowly decrease and switch medium.
4. Keep both primary and secondary antibodies sterile. Live cells will turn over the antigen; hence, staining will disappear after 24 h.
5. Cell needs to form spheres. Rough resuspension will create single-cell suspension, and cells will not form spheres.

6. Ultralow attachment dish will prevent attachment of EB to dish.
7. EB Medium does not contain bFGF which prevent cells from maintaining in pluripotent state and induce spontaneous differentiation.
8. The reprogramming plasmids are normally lost during iPSC proliferation and therefore not present around passage 10>.

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Modeling Genomic Imprinting Disorders Using Induced Pluripotent Stem Cells

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Abstract

Induced pluripotent stem cell (iPSC) technology has allowed for the invaluable modeling of many genetic disorders including disorders associated with genomic imprinting. Genomic imprinting involves differential DNA and histone methylation and results in allele-specific gene expression. Most of the epigenetic marks in somatic cells are erased and reestablished during the process of reprogramming into iPSCs. Therefore, in generating models of disorders associated with genomic imprinting, it is important to verify that the imprinting status and allele-specific gene expression patterns of the parental somatic cells are maintained in their derivative iPSCs. Here, we describe three techniques: DNA methylation analysis, allele-specific PCR, and RNA FISH, which we use to analyze genomic imprinting in iPSC models of neurogenetic disorders involving copy number variations of the chromosome 15q11–q13 region.

Keywords: Induced pluripotent stem cells, Genomic imprinting, DNA methylation, Allele-specific PCR, RNA FISH

1 Introduction

Genomic imprinting is an epigenetic phenomenon by which genes are expressed in a parent-of-origin dependent manner. This process occurs primarily in eutherian mammals, although it can also be observed in plants and metatherians. The estimated number of imprinted genes varies from a conservative estimate of 100 genes (1) to more than 1,000 (2), depending on how such imprinted expression was ascertained and the criteria for determining whether a gene is imprinted (3).

DNA cytosine methylation as well as specific active and repressive histone modifications are involved in mediating the allele-specific gene expression in genes regulated by genomic imprinting. Most imprinted loci have an imprinting control region (ICR) that is an area of differential DNA methylation between the two parental alleles (1). The ICR is typically methylated on the silent, repressed allele and unmethylated on the expressed, active allele. Repressive histone modifications, such as trimethylation of histone H3 on

lysine 9 (H3K9me3) and histone H3 lysine 79 (H3K79me3), often accompany DNA methylation on the repressed allele of the ICR (4). Active histone modifications such as di- and trimethylation of histone H3 on lysine 4 (H3K4me2 and me3), mono- and dimethylation of histone H3 lysine 79 (H3K79me1 and me2), and acetylation at histone H4 lysine 91 (H4K91Ac) often adorn the unmethylated allele at the ICR (4). Altogether, the ICR often controls the imprinted expression of all of the genes within the imprinted cluster, regardless of the parent of origin of their gene expression. Thus, the ICR harbors important epigenetic modifications that ultimately determine the allele-specific expression of several imprinted genes within a cluster.

Imprinted genes, which are expressed from a single parental allele, are functionally haploid in the organism. Deletion or mutation of the single expressed allele leaves an organism null for the imprinted gene. Several human neurogenetic disorders arise from the disruption of the expressed alleles of imprinted genes. Prader-Willi and Angelman syndromes are caused by the loss of function from paternally and maternally inherited alleles of the chromosome 15q11–13 region, respectively (5). Beckwith-Wiedemann syndrome is caused by the loss of function from maternal chromosome 11p15 (6). Silver-Russell syndrome is caused by disruption of imprinted genes on chromosomes 7 (7) and 11 (6). Pseudohypoparathyroidism (Albright's hereditary osteodystrophy) and uniparental disomy 14 are also disorders caused by disruption of imprinted genes (8). Complex genetic regulation underlies the imprinted genes in each of these disorders, making it difficult to generate cell culture or animal models.

Human induced pluripotent stem cells (iPSCs) are becoming an attractive approach to modeling complex genetic disorders, such as those involving genomic imprinting (9). The use of somatic cells derived from patients enables the genetic complexities to be captured in indefinitely self-renewing stem cells that are capable of differentiation into virtually any lineage. Using iPSC technology, copy number variation and uniparental disomy that often leads to disorders involving genomic imprinting can be recapitulated in stem cells. Our group has generated iPSCs from individuals with Prader-Willi syndrome, Angelman syndrome, and Dup15q syndrome, which all involve copy number variation at an imprinted locus (10–12). However, epigenetic modifications are erased and established during the reprogramming process involved in the establishment of iPSC lines. Since appropriate expression of imprinted genes depends on the maintenance of epigenetic modifications during the reprogramming process, extensive analysis of the relevant epigenetic modifications and gene expression is a stringent requirement for modeling disorders that involve genomic imprinting. Here we detail methods that our lab uses to verify appropriate imprinted expression of genes at the human chromosome 15q11–13 locus.

Differential DNA methylation at the ICR underlies virtually every genomic imprint. We use a quantitative PCR (qPCR) based assay to quantify the fraction of methylated and unmethylated alleles at the human Prader-Willi syndrome imprinting center (PWS-IC). This assay is excellent for quantifying cytosine methylation at ICRs that are relatively stable. It can assay up to seven CpG dinucleotides, but it cannot assay the CpGs individually. In contrast to other protocols for methylation-specific PCR, this approach does not require bisulfite conversion to assay methylation at specific CpGs. Rather, it relies on the digestion of DNA by methylation-sensitive and methylation-dependent restriction enzymes followed by quantification of the DNA by qPCR (13–15). The methylation-sensitive enzymes (MSREs) used can only cleave unmethylated DNA since methylated cytosines block enzyme activity. In contrast, methylation-dependent restriction enzymes (MDREs) will only digest DNA at methylated cytosine residues. Therefore, digestion with MSREs leaves only methylated DNA and digestion with MDREs leaves only unmethylated DNA available for PCR amplification. Using primer sets designed to flank the specific DNA region of interest, the digested DNA is then analyzed by qPCR. By comparing the cycle threshold (Ct) values generated for each of the digest conditions, the relative amounts of methylated and unmethylated DNA in the region are calculated. Two control reactions are included to allow for more accurate quantitation. A mock digest is performed without either enzyme to quantify the amount of total input DNA amplified with the chosen primer set. A double digest, including both the MSREs and the MDREs, represents background signal from the qPCR reaction as all of the DNA should be digested and unavailable for amplification.

The actual expression of imprinted genes is the single most important factor in determining whether the genomic imprint is correctly established in iPSCs. The collection of epigenetic modifications amalgamates in a precise parent-of-origin gene expression pattern for each imprinted gene. We use allele-specific PCR and/or RNA fluorescence in situ hybridization (RNA-FISH) to determine allele-specific gene expression. The allele-specific PCR assay can correctly ascribe gene expression to a single parental allele. This can be used to ascertain the allele specificity across a population of cells. This assay takes advantage of polymorphic sequences on each allele to distinguish the origin of sense and anti-sense RNA transcripts expressed from the same genomic locus. The first step in this approach is to identify existing polymorphism in the target gene. Once the polymorphism is verified, strand-specific reverse transcription is used to distinguish sense and anti-sense transcripts. Followed by PCR amplification across the verified polymorphism, the origin of sense and anti-sense transcripts can be deciphered. RNA-FISH, on the other hand, allows one to assay sense versus antisense transcription, which often accompanies imprinted gene expression, on a cell-by-cell basis.

2 Materials

2.1 DNA Methylation Analysis Components

1. Cell lysis buffer: 0.5 % Sarcosyl, 200 nM NaCl, 10 mM EDTA, 10 mM Tris pH 8.0.
2. Proteinase K (20 mg/mL, catalog #P8107S, New England Biolabs).
3. Cell scrapers.
4. 1.5 mL Eppendorf tubes.
5. Phenol-chloroform with isoamyl alcohol.
6. 100 % ethanol.
7. DNase-free distilled water (dH_2O).
8. Microcentrifuge.
9. NanoDrop spectrophotometer.
10. EpiTect Methyl II DNA Restriction Kit (catalog #335452, SABiosciences) or individual MSREs/MDREs (e.g., MspI, HpaII, etc., New England Biolabs).
11. 0.2 mL PCR microtube strips with caps.
12. Thermocycler.
13. EpiTect Methyl PCR Primer Assay for the gene of interest. For the PWS-IC, we use a human SNRPN primer (catalog #EPHS104317-1A, SABiosciences). Alternatively, primer sets can be designed that flank the methylation sites to be analyzed using various software programs and ordered from commercial providers.
14. SYBR-Green PCR Master Mix (catalog #330520, SABiosciences or catalog #4334973, Applied Biosystems).
15. 96-well PCR plates.
16. Tabletop centrifuge.
17. Quantitative PCR instrument.

2.2 Allele-Specific PCR Components

1. Primers for amplifying polymorphic sequence.
2. Phusion® High-Fidelity DNA Polymerase (catalog #M0530S, New England Biolabs).
3. QIAquick PCR Purification Kit (catalog #28104, Qiagen).
4. T7 Endonuclease I (catalog #M0302S, New England Biolabs).
5. Gel electrophoresis apparatus, 2 % agarose gel, and matching buffer.
6. RNA-Bee (catalog #CS-104B, Tel Test, Inc.).
7. TURBO DNA-free Kit (catalog #AM1907, Life Technologies).
8. Superscript III (catalog #18080044, Life Technologies).

9. Tagged RT primers (S-RT and ATS-RT).
10. Advantage 2 Polymerase Mix (catalog #639207, Clontech).
11. PCR primers for tag sequences (F′ and R′).
12. PCR forward and reverse primers (F and R).
13. Novex TBE 6 % polyacrylamide gel (catalog #EC6265BOX, Life Technologies).
14. TBE Buffer (catalog #15581-044, Life Technologies).
15. XCell SureLockTM Mini-Cell (catalog #EI0001, Life Technologies).
16. SYBR Gold (catalog #S-11494, Life Technologies).

2.3 RNA FISH Components

1. 12 mm round coverslips (catalog #12-545-80, Fisherbrand).
2. Nick Translation DNA Labeling System (catalog #ENZ-42910, Enzo).
3. ChromaTide Alexa Fluor 594-5-dUTP (catalog #C-11400, Life Technologies).
4. SNORD115 BAC (RP11-37A4, CHORI).
5. MAXIscript T7/T3 Kit (catalog #AM-1324M, Life Technologies).
6. ChromaTide Alexa 488-5-UTP (catalog #C-11403, Life Technologies).
7. Not I (catalog #R0189L, New England Biolabs).
8. Carbonate buffer: 60 mM Na_2CO_3 and 40 mM $NaHCO_3$.
9. Micro Bio-Spin P-30 Tris column (catalog #732-6250, Bio-Rad).
10. Human Cot-I DNA 1 μg/μL (catalog #1 581 074, Roche).
11. Salmon sperm DNA.
12. 20× SSC (Various SSC dilutions, including 4× SSC, 2× SSC, and 1× SSC, are made from this 20× SSC stock.)
13. 2× hybridization buffer: 1 part 20× SSC, 1 part 10 mg/mL BSA, 1 part nuclease-free water, and 2 parts 50 % dextran sulfate.
14. CSK buffer: 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, and 10 mM PIPES pH 6.8.
15. 1 % Triton X-100/CSK: 100 μL Triton X-100 in 9.9 mL CSK buffer.
16. 50 % formamide/2× SSC: 1 part 100 % formamide in 1 part 4× SSC.
17. Vectashield with DAPI (catalog #H-1200, Vector Laboratories).

3 Methods

3.1 DNA Methylation Analysis

3.1.1 Isolate Genomic DNA

1. Collect iPSCs (approximately 2×10^6 cells) by scraping with a cell scraper and pellet in a 1.5 mL Eppendorf tube.
2. Add 300 μL of Cell Lysis Buffer and 20 μL of 20 mg/mL Proteinase K to pelleted cells. Vortex and incubate in a 55 °C water bath overnight (at least 18 h) (*see* **Note 1**).
3. Add 300 μL of phenol-chloroform to lysate and vortex at least 30 s.
4. Spin for 3 min at maximum speed in a room temperature microcentrifuge.
5. Carefully transfer the upper aqueous phase to a new 1.5 mL Eppendorf tube. Avoid contact with the lower phenol phase or the white interphase which may have formed after centrifugation.
6. Add 1 mL of 100 % ethanol to aqueous phase and invert tube several times. White strands of DNA should be visible as it precipitates.
7. Spin for 5 min at maximum speed in a room temperature microcentrifuge.
8. Remove all supernatant and air-dry pelleted DNA for approximately 5–10 min.
9. Resuspend DNA in 50–100 μL of dH_2O.
10. Check concentration of DNA using a NanoDrop spectrophotometer.
11. Store at 4 °C or at −20 °C for long-term storage.

3.1.2 Perform Restriction Digest with MSREs and MDREs

1. Prepare a solution of 0.5–1.0 μg of DNA with the appropriate dilution of restriction digest buffer and dH_2O. If using the EpiTect Methyl II DNA Restriction Kit, use 26 μL of 5× Digestion Buffer and add dH_2O to a final volume of 120 μL. If using restriction enzymes from another source, prepare a volume of 200 μL, which is sufficient for four 50 μL individual restriction digest reactions (*see* **Note 2**).
2. Thoroughly mix the DNA/buffer solution and divide equally into four separate PCR tubes (*see* **Note 3**).
3. Set up the restriction digest reactions and controls by adding the following to each PCR tube: If using the EpiTect Methyl II DNA Restriction Kit, for MSRE reaction—add 1 μL of dH_2O and 1 μL of MSRE (Enzyme A), for MDRE—add 1 μL of dH_2O and 1 μL of MDRE (Enzyme B), for the double digest—add 1 μL each of the MSRE and MDRE, and for the mock digest—add 2 μL of dH_2O in place of restriction enzymes (*see* **Note 4**).

4. Mix reactions gently by pipetting and briefly spin samples in a microcentrifuge.
5. Incubate reactions at 37 °C in a thermocycler for at least 6 h to overnight.
6. Inactivate restriction enzymes by incubating reactions at 65 °C for 20 min in a thermocycler.
7. Perform qPCR with digested DNA or store digest reactions at −20 °C until use.

3.1.3 Analyze Digested DNA by qPCR

1. Prepare qPCR reaction master mix by combining SYBR-green qPCR Master Mix, EpiTect Methyl PCR Assay Primer, and dH_2O sufficient to run each DNA sample in triplicate (each mock, MSRE, MDRE, and MSRE + MDRE digest is considered a separate sample). To account for pipetting volume error, prepare enough master mix for at least two excess reactions. Each 20 μL total PCR reaction will ultimately contain 10 μL SYBR-green qPCR Master Mix, 1 μL EpiTect Methyl PCR Assay Primer, 5 μL of digest DNA, and 4 μL dH_2O (*see* **Note 5**).
2. Pipette 15 μL of master mix reaction into each well of the qPCR plate. Add 5 μL of sample to the individual wells and mix my gentle pipetting.
3. Seal the qPCR plate and spin briefly in a tabletop centrifuge.
4. Perform qPCR amplification. If using EpiTect Methyl PCR Assay primers the following conditions should be used: (1) 95 °C for 10 min to activate HotStart DNA polymerase. (2) 3 cycles of 99 °C for 30 s and 72 °C for 1 min. (3) 40 cycles of 97 °C for 15 s and 72 °C for 1 min. (4) Standard melting curve. Record SYBR-green signal at each of the 72 °C cycles in step 3 (*see* **Note 6**).
5. Calculate percent methylated and percent unmethylated DNA using the Ct values generated from the qPCR run. SABiosciences provides an Excel spreadsheet which can be used to automatically perform these calculations after inserting the mean Ct value for each sample. This spreadsheet takes into account the percentage of DNA resistant to digestion (represented by the double digest reaction) in calculating the percent methylated and unmethylated DNA. Alternatively, several methods have been independently reported to perform these calculations (*see* **Note 7**).

3.2 Allele-Specific PCR

3.2.1 Screening for Polymorphic Sequence

1. Use tracks under the categories of "Variation" or "Repeats" in UCSC Genome Browser (genome.ucsc.edu) to look for reported SNPs or short tandem repeats in the target gene. As an example, a polymorphic region of short tandem repeats in an intron of UBE3A gene was identified using the "Microsatellite" track in Fig. 1.
2. Carry out T7 E1 assay with the following steps modified from the manufacturer's protocol for T7 Endonuclease I.
   (a) Prepare good quality genomic DNA from cells as described in Section 3.1.1 (*see* **Note 8**).
   (b) Design primers to amplify about 1 kb region around the polymorphic sequence, preferably with the polymorphic sequence offset from the center as shown in Fig. 1 (*see* **Note 9**).
   (c) Carry out 50 μL PCR reaction using Phusion® High-Fidelity DNA Polymerase or other proofreading DNA polymerase to obtain correct PCR product.
   (d) Clean up PCR product using QIAquick PCR Purification Kit and elute DNA in 30 μL elution buffer. When eluting, let the column sit in elution buffer at room temperature for at least 5 min before spinning to increase yield.
   (e) Measure DNA concentration using a NanoDrop spectrophotometer (*see* **Note 8**).
   (f) Add 150 ng of cleaned PCR product into a 0.2 mL PCR tube. Add 1 μL of 10× NEBuffer 2 and bring the total volume up to 10 μL with water. Save the remaining PCR product as a control for gel electrophoresis in a later step.
   (g) Run the following program in a thermocycler to make heteroduplex:

95 °C	10 min
95–85 °C	Decrease 2.0 °C per second
85 °C	1 min

(continued)

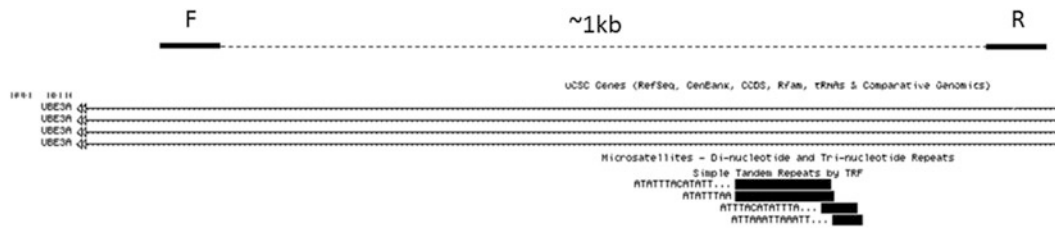

Fig. 1 Short tandem repeats in an intron in *UBE3A* gene shown in USCS genome browser. Ideal PCR primers for T7 E1 assay is depicted on *top* (*F* forward primer, *R* reverse primer)

(continued)

85–75 °C	Decrease 0.3 °C per second
75 °C	1 min
75–65 °C	Decrease 0.3 °C per second
65 °C	1 min
65–55 °C	Decrease 0.3 °C per second
55 °C	1 min
55–45 °C	Decrease 0.3 °C per second
45 °C	1 min
45–35 °C	Decrease 0.3 °C per second
35 °C	1 min
35–25 °C	Decrease 0.3 °C per second
25 °C	1 min
4 °C	∞

(h) Add 0.5 μL of T7 Endonuclease I into the PCR tube and incubate at 37 °C for 30 min in a thermocycler to cut any heteroduplex that has formed in the previous step.

(i) Stop the reaction by adding 2.5 μL of loading dye that contains 0.1 M EDTA (*see* **Note 10**).

(j) Run the digested product alongside the original PCR product from step f on a 2 % agarose gel. As an example, a gel for a polymorphic sequence verified by T7 E1 assay is shown in Fig. 2.

3.2.2 RNA Isolation and DNase Treatment

1. Collect and pellet cells by centrifugation in a 1.5 mL Eppendorf tube.
2. Follow manufacturer's protocol (16) using RNA-Bee (*see* **Note 11**) with the following modifications: (1) For phase separation, shake tube vigorously for 30 s. (2) For RNA precipitation, let tube sit at room temperature for 10 min before centrifuging at 4 °C for 15 min. (3) For RNA wash, centrifuge tube at 4 °C for 10 min. (4) For RNA solubilization, dissolve pellet in nuclease-free water (*see* **Note 12**).
3. To reduce genomic DNA contamination, treat isolated RNA from the previous step with DNaseI using TURBO DNA-free Kit. Follow manufacturer's protocol exactly, except extend the 37 °C incubation time to 45 min.
4. Measure the RNA concentration using a NanoDrop spectrophotometer.
5. Store RNA at −80 °C until ready to use for reverse transcription.

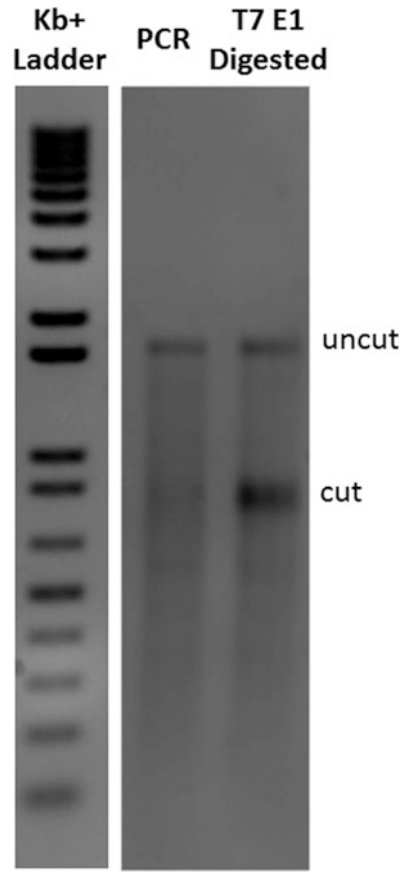

Fig. 2 A polymorphism in *UBE3A* gene verified by T7 E1 assay in a patient iPSC cell line. The PCR product is around 1.7 kb, with the polymorphic sequence located at the center (*left lane*). The non-perfectly matched DNA resulted from the polymorphism is cut by T7 Endonuclease I and leads to an 850 bp band (*right lane*)

3.2.3 Primer Design for Strand-Specific Reverse Transcription (RT) and PCR

1. Design the following primers surrounding the polymorphic sequence as depicted in Fig. 3 (*see* **Notes 13–16**)
   (a) *Tagged RT Primers for the sense transcript (S-RT) and antisense transcript* (*ATS-RT*): These primers consist of two parts: a 5′ tag and a gene-specific RT primer. For the 5′ tag on S-RT, use the following sequence as a template: 5′-CAGTCGGGCGTCATCA-3′ (17). For the 5′ tag on ATS-RT, use the following sequence from M13R as a template: 5′-GGAAACAGCTATGACCAT-3′. Add or remove nucleotides from the ends of the primer sequence to optimize the melting temperature and minimize secondary structure formation (*see* **Note 17**). For the gene-specific RT primers, use Primer3 (18, 19) or other similar software to design a primer located upstream of the polymorphic

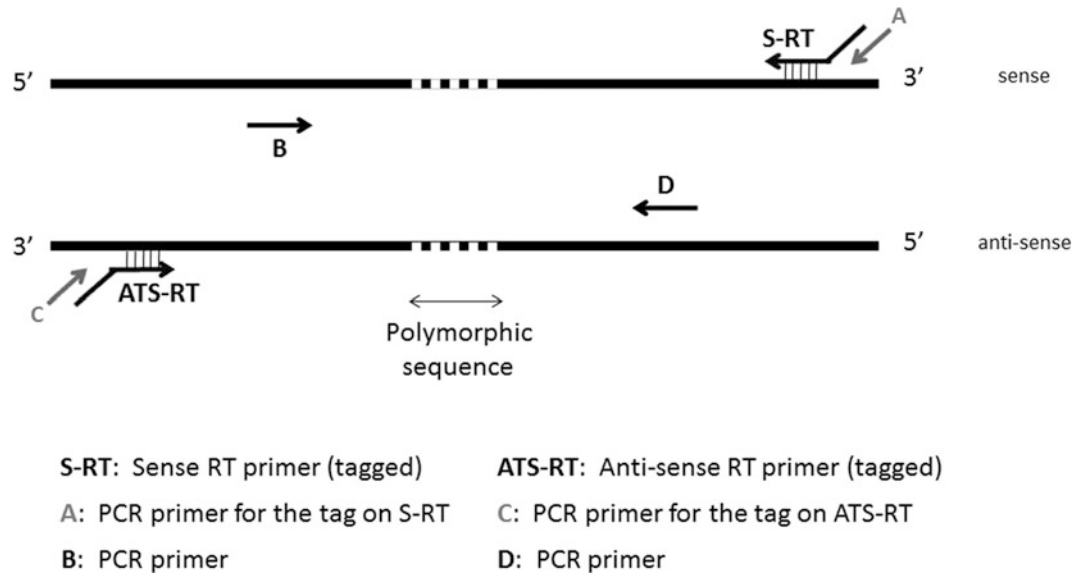

Fig. 3 Primer designs for strand-specific reverse transcription and the following PCR amplification. The *arrow head* in each primer points towards the primer's 3′ direction

sequence on the sense and anti-sense transcripts. The melting temperature for these sequences should be around or slightly below 55 °C (*see* **Note 18**). Check the tag sequence, the gene-specific RT primer sequence, and the final combined sequence using BLAST or UCSC genome browser for mis-priming.

(b) *PCR primer for the tag sequence on S-RT* (*A*): This primer is the same sequence as the tag sequence on S-RT.

(c) *PCR primer for the tag sequence on ATS-RT* (*C*): This primer is the same sequence as the tag sequence on ATS-RT.

(d) *PCR primer B*: This primer is located upstream of the polymorphic sequence on the sense transcripts.

(e) *PCR primer D*: This primer is located upstream of the polymorphic sequence on the anti-sense transcripts.

3.2.4 Strand-Specific Reverse Transcription (RT)

1. Follow manufacturer's protocol for Superscript III.
2. Set up two RT reactions: one using S-RT as the gene-specific primer and the other using ATS-RT as the gene-specific primer. For each reaction, include a no RT control that contains the same ingredients except for the reverse transcriptase. The no RT controls (S-noRT and ATS-noRT) will indicate any genomic DNA contamination in the following PCR reaction.

3. Heat the primer/dNTP/DNase-treated RNA mixture in a thermocycler at 65 °C for 5 min and cool on ice for at least 1 min.
4. After adding the remaining reaction components as instructed, place the tubes in a thermocycler and run the following program for reverse transcription: 25 °C for 5 min, 55 °C for 60 min, and 70 °C for 15 min.
5. Keep the tubes on ice or store at −20 °C until ready to use for the allele-specific PCR.

3.2.5 *Allele-Specific PCR*

1. Follow manufacturer's protocol for Advantage 2 Polymerase Mix (or other high-fidelity proofreading DNA polymerase).
2. Set up two sets of PCR reactions for each S-RT, ATS-RT, S-noRT, ATS-noRT, as well as a genomic DNA control (*see* **Note 19**). For the first set, use A and B as PCR primers. These two primers amplify the sense transcripts specifically. For the second set, use C and D as PCR primers which will specifically amplify the anti-sense transcripts. The PCR result with different primer combinations is summarized in Fig. 4a.
3. Once the PCR result is verified with strand-specific primers as stated above, you can carry out a PCR using primers B and D to obtain an image that is easier to interpret visually as shown in Fig. 4b (*see* **Note 20**).

a

PCR Primers	S-RT	ATS-RT	gDNA	S-noRT	ATS-noRT
A and B	✔	✖	✖	✖	✖
C and D	✖	✔	✖	✖	✖
B and D	✔	✔	✔	✖	✖

b

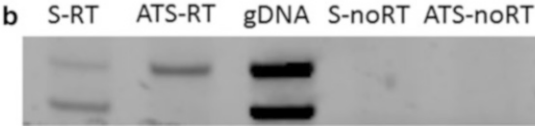

Fig. 4 Allele-specific PCR for sense and anti-sense transcripts across polymorphism. (**a**) The summary of PCR amplification results with different primer combinations. (**b**) This gel shows allele-specific PCR using primer B and D across the polymorphism identified in Fig. 2. The data indicates that the allele that expresses UBE3A-ATS transcripts expresses UBE3A sense transcripts at a lower level than the other allele. Genomic DNA serves as a positive control for PCR amplification (S-RT: reverse transcription using S-RT primer; ATS-RT: reverse transcription using ATS-RT primer; gDNA: genomic DNA; S-noRT: no reverse transcriptase control for RT using S-RT primer; ATS-noRT: no reverse transcriptase controls for RT using ATS-RT primer)

3.3 RNA FISH

3.3.1 Plate iPSC Colonies on Coverslips

1. Plate irradiated mouse embryonic fibroblasts (MEFs) onto 12 mm round coverslips 1–2 days before seeding iPSC colonies on them.
2. Cut iPSC colonies mechanically and place them on MEF-coated coverslips and let them grow in human embryonic stem cell medium for 4–6 days.

3.3.2 Make SNORD115 BAC Probes Using Nick Translation DNA Labeling System (see **Note 21**)

1. Dilute Alexa Fluor 594-5-dUTP to 0.3 mM (10 μL of 1 mM Alexa Fluor 594-5-dUTP in 23.3 μL of nuclease-free water). Keep the unused portion in −20 °C and avoid light.
2. Prepare diluted DNase I freshly (80 μL of 1× DNase dilution buffer and 1 μL of DNase I). Gently flick to mix. DO NOT VORTEX!!!
3. Add the following reagents from the Nick Translation DNA Labeling system accordingly.

SNORD115 BAC	1 μg (Adjust volume to 25 μL with nuclease-free water)
Reaction buffer	5 μL
dNTP Mix	5 μL
dTTP	2.5 μL
0.3 mM Alexa 594-dUTP	2.5 μL
DNA polymerase I	5 μL
Fresh diluted DNase I	5 μL
Total volume	50 μL

4. Carefully mix the reagents by flicking and briefly centrifuge.
5. Incubate the mixture for 2 h at 15 °C.
6. After incubation, place the reactions on ice.
7. Terminate the reaction by adding 5 μL of Stop Buffer and heat up for 5 min at 65 °C.

Pause point: Labeled BAC probes can be stored at −20 °C prior to use.

3.3.3 Make UBE3A Riboprobes Using MAXIscript T7/T3 Kit (See **Note 22**)

1. Clone UBE3A cDNA C7-3 (20) into pBluescript SK+.
2. Linearize the plasmid with Not I (*see* **Note 23**).
3. Add the following reagents from the MAXIscript T7/T3 kit accordingly.

Linearized UBE3A plasmid	1 μg
10× Transcription buffer	2 μL
10 mM ATP	1 μL

(continued)

(continued)

10 mM CTP	1 μL
10 mM GTP	1 μL
10 mM UTP	1 μL
1 mM Alexa 488-UTP	2.5 μL
T7 or T3 Enzyme (*see* **Note 24**)	2 μL
Nuclease-free water	to 20 μL

4. Carefully mix the reagents by flicking and briefly centrifuge.
5. Incubate the mixture for 1 h at 37 °C.
6. Add 1 μL of DNase I and incubate at 37 °C for 15 min to remove any residual DNA.

   Pause point: Labeled riboprobes can be stored at −20 °C prior to use.
7. Take 5 μL of in vitro transcribed riboprobes and hydrolyze it by adding 20 μL carbonate buffer and 20 μL of nuclease-free water (*see* **Note 25**).
8. Incubate at 60 °C for 30 min to obtain the optimal size of riboprobes (*see* **Note 26** and Fig. 5).

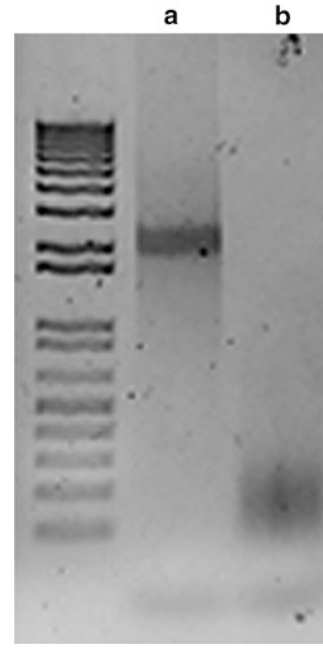

Fig. 5 Gel electrophoresis of riboprobes. (**a**) In vitro transcribed and labeled riboprobes. (**b**) Hydrolyzed riboprobes. DNA ladder is used to approximately estimate the size of riboprobes

9. Neutralize with 2.5 μL (or 1/20 volume) of 10 % acetic acid.
10. Purify riboprobes by running through a Micro Bio-Spin P-30 Tris column to remove the unlabeled Alexa Fluor 488-5-UTP.

3.3.4 Precipitate Probes

1. Add 5 μL of labeled SNORD115 BAC probes to the purified riboprobes (*see* **Note 25**).
2. Add 5 μL of Cot-1 DNA and 5 μL of salmon sperm DNA to the combined probes.
3. Add 2.5× volume of 100 % ice-cold ethanol to the mix.
4. Mix well and precipitate the probes for 30 min at −20 °C.
5. Centrifuge at 12,000 rpm for 30 min at 4 °C.

   (While waiting for the centrifuge, prepare coverslips and 2× hybridization buffer, *see* Section 3.3.5)
6. Carefully remove the supernatant and air dry in the dark for at least 15 min.

3.3.5 Prepare Coverslips and 2× Hybridization Buffer

1. Aspirate iPSC medium and wash with PBS once.
2. Incubate the coverslips with ice-cold CSK buffer for 30 s at room temperature.
3. Incubate the coverslips with ice-cold 1 % Triton X-100/CSK buffer for 5–10 min at room temperature (*see* **Note 27**).
4. Incubate the coverslips with ice-cold CSK buffer for 30 s.
5. Fix the coverslips with 4 % paraformaldehyde/PBS for 10 min at room temperature.

   Pause point: The coverslips can be stored in 70 % ethanol at 4 °C until use.
6. Dehydrate the coverslips with 85 %, 95 %, and 100 % ethanol for 2 min each.
7. Air-dry for 10–15 min until everything evaporates.
8. Make 2× hybridization buffer.

3.3.6 Hybridization

1. Resuspend the dry pellet in 5 μL 100 % formamide and denature the probes at 90–95 °C for 10 min (for two coverslips).
2. Meanwhile, create a humid chamber with 50 % formamide/2× SSC.
3. Add equivalent amount of 2× hybridization buffer to denatured probes, mix well, and spin it down.
4. Wrap a slide with parafilm and apply ~5 μL of denatured probes on the film (avoid bubbles).
5. Put coverslips upside down and make sure iPSC colonies are well covered.
6. Place the slide in the humid chamber and incubate at 37 °C overnight.

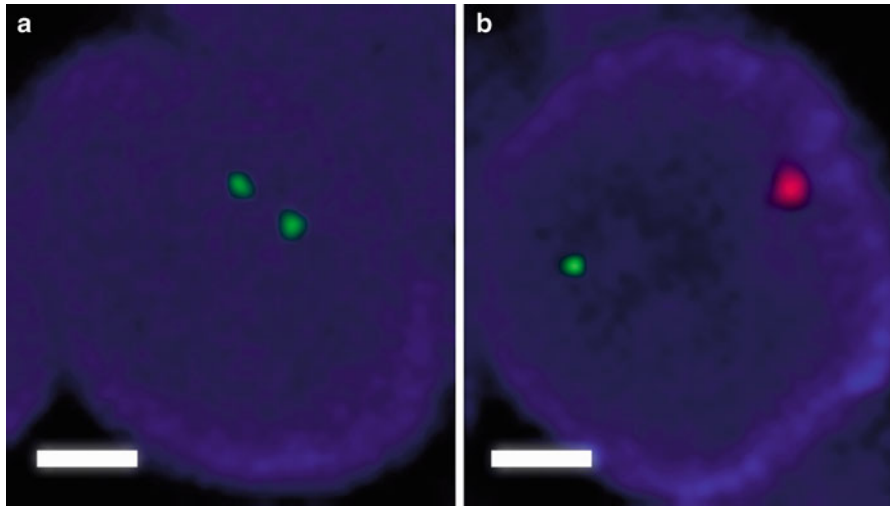

Fig. 6 Representative RNA-FISH images. (**a**) A normal iPSC with UBE3A (*green*) transcribed from both alleles. SNORD115 (*red*) is not expressed in normal iPSCs. (**b**) The paternal UBE3A is silenced due to aberrant SNORD115 expression in the atypical Prader-Willi Syndrome iPSCs. Scale bar: 2 μm

3.3.7 Posthybridization Wash

1. Take the coverslip and put the side with iPSC colonies up on a new parafilm wrapped slide.
2. Wash with 50 % formamide/2× SSC three times at 39 °C for 7 min.
3. Wash with 2× SSC three times at 39 °C for 7 min.
4. Wash with 1× SSC two times at 39 °C for 7 min (wash more or less with 1× SSC to have best signal/noise ratio).
5. Air-dry the coverslips in the dark until everything evaporates.
6. Mount it with 3–4 μL Vectashield with DAPI and seal it.
7. The coverslips are ready for imaging (*see* Fig. 6).

4 Notes

1. Alternatively, any commercially available kit can be used to isolate high-quality genomic DNA.
2. We often reduce the reaction volume by half when performing this assay using the EpiTect Methyl II Restriction Enzyme kit. This results in 15 μL of each digested DNA which is sufficient to run triplicate qPCR technical replicates using 5 μL of DNA per qPCR reaction.
3. It is very important to ensure that each digest tube contains the same amount of DNA.

4. If using MSREs and MDREs from another source, follow manufacturer guidelines for running a standard restriction digest with appropriate volumes of enzyme.
5. If using PCR primers other than the EpiTect assay, adjust volume of water to accommodate volume of PCR primers used.
6. If using primer sets other than an EpiTect assay, qPCR conditions such as annealing temperature will need to be optimized.
7. According to Bruce et al. (14), in order to calculate percent methylation, first subtract the mean Ct value of the Mock digest from the mean Ct value of the MSRE reaction to yield value *x*. Then use the equation: % meth = $100 \times 1/P^{M}$ where $P^{M} = 2^{x}$. Oakes et al. (15) use a slightly modified equation. Again, subtract the mean Ct of the Mock digest from the mean Ct of the MSRE or MDRE reaction to yield ΔCt. Then using the ΔCt of MSRE, use the equation: % meth = $100 \times (e^{-0.7(\Delta Ct)})$ or using the ΔCt of MDRE, use the equation: % meth = $100 \times (1-e^{-0.7(\Delta Ct)})$.
8. DNA with good quality has a 260/280 ratio of 1.8–2.0 when measured by a NanoDrop spectrophotometer.
9. If there are known heterozygous and homozygous regions for polymorphism, it is a good practice to include a positive and a negative control for the T7 E1 assay.
10. Alternatively, add 0.5 μL of 0.5 M EDTA if loading dye does not contain EDTA.
11. Alternatively, use any RNA isolation kit that produces high-quality RNA. Note the 260/280 ratio when measuring RNA concentration with a NanoDrop spectrophotometer. High-quality RNA extracted by RNA-Bee typically has a ratio of 1.6–1.9.
12. Do not use DEPC-treated water as it may not be compatible with following experiments.
13. The RT primers should be within a few hundred nucleotides of the polymorphic region for reverse transcription, yet far enough from the polymorphic region to accommodate the PCR primers downstream.
14. For ease of analysis, the polymorphic region should preferably be located at the center of all three primer pairs so that the resulting PCR products from sense and anti-sense strand are similar in size for each allele.
15. The four PCR primers (A, B, C, and D) should have similar melting temperature so that they can be used interchangeably during PCR.
16. If the polymorphism is a single nucleotide change or the difference in repeat numbers is too small to be resolved using gel

electrophoresis, sequencing is required to decipher the origin of the sense and anti-sense transcripts. In this case, PCR primers B and D can be used as sequencing primers and should be located at least 40 nucleotides away from the polymorphic region to avoid the initial low-quality sequencing reads.

17. As PCR primers A and C are dependent on the tag sequences in the RT primers, it is important to check melting temperatures and secondary structures of the tag sequences. Software such as Primer Express® 3.0 is recommended for this purpose. Ideally, these sequences should be unique and do NOT align to the host genome to avoid nonspecific PCR amplification.
18. The melting temperature of gene-specific RT primer sequences should be similar to the optimal working temperature of the reverse transcriptase used. According to manufacturer's protocol, Superscript III works the best at 55 °C for gene-specific reverse transcription.
19. When the polymorphic sequence is not centered, the band size between PCR product from sense and anti-sense specific PCR amplification will be different. If your polymorphism leads to size differences between two alleles, it may be slightly harder to interpret visually on a gel. In this case, try carrying out PCR using B and D. This primer set is not as specific and may give you false result if the reverse transcription step is not specific enough. Other than changing the band size, it should give you similar result in terms of number of bands and intensity as using strand-specific primers.
20. It is advised to do a serial dilution for the genomic DNA control to match the band intensity to a similar level as the other samples.
21. The following steps are adapted from Nick Translation DNA Labeling System protocol (catalog #ENZ-42910, Enzo).
22. The following steps are partially adapted from MAXIscript T7/T3 Kit protocol (catalog #AM-1324M, Life Technologies) and Cold Spring Harbor protocol (21).
23. A restriction enzyme that cuts downstream of the template strand of the cDNA plasmid should be used to linearize your plasmid. If the plasmid DNA is not linearized completely, heterogeneous and extremely long RNA transcripts will be generated due to the processiveness property of RNA polymerases.
24. Depending on how cDNA is cloned into your vector plasmid, T3 or T7 enzyme would be used to produce the right orientation of riboprobes. For example, if cDNA is cloned in the T3–T7 direction, T7 enzyme would be used to generate riboprobes that would detect sense transcript.

25. The volume of riboprobe and BAC probe used can be adjusted to obtain optimal FISH signal.
26. Run 1 μL of in vitro transcribed riboprobes (product from Section 3.3.3, **step** 6) and 5 μL hydrolyzed riboprobes (product from Section 3.3.3, **step** 8) on a 1 % agarose gel to obtain optimal size for hybridization. The optimal probe size should be less than 250 nucleotides. Larger size of probes causes low penetration of probes. The incubation time can be calculated using the following formula: $t = (L_0 - L_1)/0.11 \times (L_0)(L_1)$, where L_0 is the original length of transcript and L_1 is the desired riboprobe length (21).
27. Increasing the incubation time of 1 % Triton X-100/CSK will increase penetration of probes into the nuclei.

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Generation and Characterization of Induced Pluripotent Stem Cells from Patients with mtDNA Mutations

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Abstract

Generation of induced pluripotent stem cells from patient cells has revolutionized disease modeling in recent years. One research area, where disease models have previously been scarce, is disorders with mutations in mitochondrial DNA. These are a common cause for human disease and often cause very tissue specific phenotypes with vast clinical heterogeneity. iPS technology has now opened up new possibilities for mechanistic studies of these diseases.

Keywords: Human induced pluripotent stem cells, iPSc, Reprogramming, mtDNA, Mitochondria, Mitochondrial disease, Disease modeling

1 Introduction

Mitochondrial respiratory chain dysfunction is a common cause of inherited human disease. Because of the dual genetic control of mitochondria, both nuclear and mitochondrial DNA (mtDNA) mutations can lead to mitochondrial disease. Mitochondrial disease most commonly manifests in tissues that depend most on oxidative energy metabolism, and the most prominent and common features in patients are usually seen in cardiac, muscle, or neural tissues, or in sensory organs. Patients with mitochondrial disease show vast clinical heterogeneity, and even a single mutation can lead to different manifestations [1]. To date, more than 200 different mtDNA mutations are known to cause human disease. However, the pathogenetic mechanisms underlying these disorders are still mostly unknown, since model systems have been scarce. Current transgenic technologies are not efficient in manipulating mitochondrial DNA, limiting the targeted engineering of mutations in mtDNA. Thus, mechanistic studies have been confined to those cell types that are freely available from patients, such as fibroblasts, and to the few mouse models generated through cybrids [2–4].

The use of induced pluripotent stem (iPS) cells [5, 6] has opened up new possibilities for the use of patient material. Generation of iPSCs from patient samples has become routine, and further

differentiation of these iPS cells allows mechanistic studies in disease relevant cell types that are otherwise not available from patients.

2 Materials

2.1 Reprogramming and iPSC Culture

Patient fibroblasts from skin biopsy (*see* **Note 1**).

Episomal plasmids from Addgene: 27077 (pCXLE-hOCT3/4-shp53-F), 27078 (pCXLE-hSK), 27079 (pCXLE-hMLN).

Feeder cells from mouse embryonic fibroblasts (MEFs). Prepared from 12.5 dpc ICR embryos, mitotically arrested with mitomycin-c treatment.

Fibroblast culture medium: DMEM (high glucose), glutamax, penicillin/streptomycin, 10 % FBS.

hES medium (used during iPSC establishment): DMEM:F12, non-essential amino acids, glutamax, 0.1 mM beta-mercaptoethanol, penicillin/streptomycin, 20 % KO-Serum Replacement (Invitrogen), and 10 ng/ml bFGF.

Essential 8 medium (used during iPSC culture, Life technologies).

Uridine.

Phosphate buffered saline (PBS), Mg^{++} and Ca^{++} free.

TrypLE Select (Invitrogen).

EDTA 0.5 mM.

Gelatin 0.1 %.

Matrigel (BD Biosciences).

Dimethylsulfoxide (DMSO).

Hemocytometer or automated cell counter.

Electroporator, e.g., Neon Transfection system (Invitrogen; Life Technologies).

Scalpels or needles.

Cell culture plates and dishes (6 cm, 6-well, 4-well).

2.2 Assessing mtDNA Copy Number

DNA lysis buffer: 100 mM Tris–HCl (pH 8.5), 5 mM EDTA, 0.2 % SDS, 200 mM NaCl, 100 μg/ml Proteinase K (freshly added).

Q-PCR master mix, e.g., iQ SYBR green supermix (Bio-Rad).

Q-PCR instrument, e.g., CFX96 Real time system (Bio-Rad).

96-well PCR plates suitable for the Q-PCR machine.

Optical adhesive covers.

Primers:

5′-GCCTGCCTGATCCTCCAAAT-3′ (CytB-F).

5′-AAGGTAGCGGATGATTCAGCC-3′ (CytB-R).

5′-TGTGTGCTCTCCCAGGTCTA-3′ (APP-F).

5′-CAGTTCTGGATGGTCACTGG-3′ (APP-R).

2.3 Characterization of the Respiratory Chain Proteins with Western Blotting

Dodecyl-B-D-maltoside (DDM).

Protease inhibitor, e.g., Complete mini (Roche).

Phosphate buffered saline (PBS), Mg^{++} and Ca^{++} free.

Cell scrapers.

Bradford reagent (Bio-Rad).

Syringe filters.

Syringes.

Bovine serum albumin (1 mg/ml).

Plate reader and 96-well plates for the plate reader.

2× Laemmli Sample Buffer: 4 % SDS, 10 % β-mercaptoethanol, 20 % glycerol, 0.004 % bromophenol blue, 0.125 M Tris–HCl pH 6.8.

Western blot running apparatus, e.g., Mini-Protean (Bio-Rad).

Running buffer: 25 mM Tris Base, 192 mM Glycine, 0.1 % SDS, pH 8.3.

SDS-PAGE gels, e.g., 4–20 % Mini-Protean TGX gels (Bio-Rad).

Transfer apparatus, e.g., Semi-dry HEP (Thermo Scientific).

Transfer Buffer: 48 mM Tris, 39 mM glycine, 10 % methanol, pH ~9.2.

Blotting paper.

Nitrocellulose or PVDF membrane.

Methanol.

TBST: 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1 % Tween 20.

Milk powder.

Primary antibodies: CI (NDUFA9; Abcam, ab14713), CII (SDHA; Abcam, ab14715), CIII (UQCRC2; Abcam, ab14745), CIV (MTCO1; Abcam, ab14705), CV (ATP5a; Abcam, ab14748), TOM20 (FL-145; Santa Cruz, sc-11415), b-tubulin (Cell Signaling, #2146).

HPR-conjugated secondary antibodies against mouse IgG and rabbit IgG.

3 Methods

In the following chapter, we describe the generation of iPSCs from fibroblasts with heteroplasmic mtDNA mutations as well as assessing mtDNA copy number and characterization of the respiratory chain proteins in these iPSCs. The generation of iPSCs can be achieved by any available reprogramming method. However, to avoid selection against heteroplasmic mtDNA mutations (meaning that the cells contain both mutated and wild-type forms of mtDNA), which renders the cells auxotrophs for uridine and pyruvate, the use of these supplements in the culture medium is important. We have successfully used both retroviral [5] and episomal plasmid [7] induction for iPSC generation and describe here the use of episomal plasmids [8]. Segregation of heteroplasmic mtDNA during reprogramming allows for derivation of disease model lines with high mtDNA mutation loads, as well as isogenic control lines with undetectable mtDNA mutation levels, from the same individual in one experiment (Fig. 1) [9]. However, in order to ensure establishment of both types of lines, several iPSC clones should always be picked from each patient line and the heteroplasmy levels of the individual clones assessed before selecting lines for further expansion.

Different functional studies can be used to characterize the established iPSCs for their mitochondrial function, and we describe here assessing mtDNA copy number and expression of the respiratory chain proteins. We typically analyze one subunit per each of the five respiratory chain complexes. Complex II is encoded solely by the nuclear genome and often shows compensatory increase, whereas the other complexes may manifest either an isolated defect

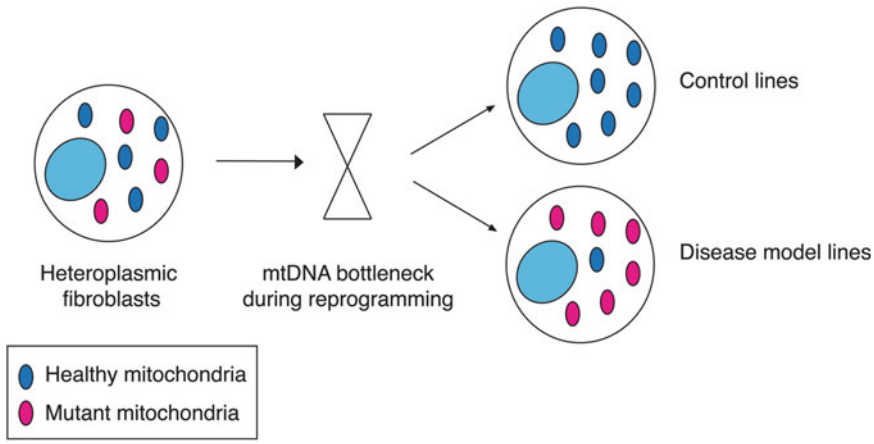

Fig. 1 Mitochondrial bottleneck during reprogramming. Segregation of heteroplasmic mtDNA during reprogramming gives rise to both disease model lines with high frequency of mutated mtDNA and isogenic control lines with undetectable levels of mutated mtDNA

of only one complex or a combined respiratory chain defect of several enzyme complexes. The RC protein amounts can be normalized against TOM20, which is a mitochondrial outer membrane protein and can be further used to assess mitochondrial mass in the cells when compared to a non-mitochondrial protein.

3.1 iPSC Generation and Culture

1. Expand low passage fibroblasts (*see* **Note 2**) in fibroblast medium. One 6 cm dish will yield around ~1–1.5 × 10^6 cells. Once the fibroblasts reach ~70 % confluency (*see* **Note 3**) and the desired cell number is reached, proceed to electroporation.
2. Prepare one 6 cm plate per cell line. Gelatinize the plate by applying gelatin (0.1 %) to the surface of the dish, aspirate, and allow to dry in biosafety cabinet. Add fibroblast medium (*see* **Note 4**) and put the plates in 37 °C, 5 % CO_2 to wait.
3. Harvest fibroblasts with TrypLE (5 min at 37 °C), collect by centrifugation at 100 × *g* for 3 min, resuspend in fresh medium, and count viable cells using hemocytometer or automated cell counter.
4. Collect 70,000 cells, wash with PBS, and resuspend in 120 μl of buffer R.
5. Mix 1 μg of each of the three plasmids pCXLE-hOCT3/4-shp53-F, pCXLE-hSK, and pCXLE-hMLN with the cells. Electroporate according to your system instructions (*see* **Note 5**). We use 1,650 V, 10 mS, and 3 pulses with the Neon electroporator (Invitrogen). Transfer the cells to the freshly prepared 6 cm dishes. Culture overnight in 37 °C, 5 % CO_2. This is considered as day 1.
6. On days 2 and 4 feed the cells with fresh fibroblast medium.
7. On day 5 prepare feeder plates for passaging the cells. Gelatinize 6-well dishes and plate ~100,000 mitotically inactivated MEFs per plate in fibroblast medium and let the cells adhere overnight in 37 °C, 5 % CO_2.
8. On day 6 split the electroporated cells to the feeder cells. Change fresh medium to the feeder cell plates. Harvest electroporated fibroblasts using TrypLE (5 min at 37 °C), collect by centrifugation at 100 × *g* for 3 min, resuspend in fresh fibroblast medium, and count viable cells. Plate 50,000–100,000 cells per well (*see* **Note 6**).
9. On day 7 change to hES medium and from there on feed every second day with fresh medium. Monitor the wells for the appearance of characteristic iPSC growth foci. These will typically become visible after 2 weeks.
10. Colonies suitable for picking may form around day 20; however clones will typically continue to arise until day 30.

11. Pick colonies mechanically, dividing large colonies into small pieces with a scalpel or a needle. Plate the picked colonies on freshly prepared 4-well dishes (*see* **Note 7**) with feeder cells and hES medium.
12. Few initial passages should be done mechanically and the cells kept on feeder cells with hES medium (*see* **Note 8**).
13. After a few passages, the cells can be transferred to Matrigel-coated plates and cultured in E8 medium using EDTA for passaging. Coat the plates with Matrigel and equilibrate to RT (1–2 h). Remove Matrigel and add E8 medium to the plates.
14. Aspirate the medium and rinse the cells with PBS. Add 0.5 mM EDTA and incubate at RT for 5–8 min. Proceed when the cells start to separate and round up. Aspirate the EDTA solution and add E8 to the dish. Remove the cells from the well by gently squirting medium and pipetting the colonies up but do not scrape the cells from the dish. Collect cells and plate to the Matrigel-coated dishes in desired ratio.
15. Grow the cells in 37 °C, 5 % CO_2 incubator and feed daily or every second day. Passage when needed.
16. Once the iPSC lines have been established, they can be frozen down. Prepare freezing medium by adding 10 % DMSO to E8 medium. Place on ice until use.
17. Treat the cells with EDTA as when splitting, but instead of medium add 1 ml of ice-cold freezing medium to each well. Remove the cells by gently squirting the colonies from the well. Collect the cells in cryovials and place on ice. Transfer first to −80 °C and after 24 h to −150 °C or liquid nitrogen for longer storage.

3.2 Assessing mtDNA Copy Number

3.2.1 DNA Extraction

1. Harvest the cells to an Eppendorf tube or lyse the cells directly on the plate.
2. Add 0.5–1 ml lysis buffer (0.5 ml/well on 24-well plate).
3. Incubate 3–5 h at 55 °C, vortex couples times during incubation.
4. Add an equal volume of isopropanol and shake until DNA precipitate becomes visible.
5. Centrifuge 12,000 × *g*, 5 min.
6. Wash the pellet with 70 % EtOH.
7. Dry 15 min at RT.
8. Resuspend in 50 μl TE or H_2O (Incubate 30 min at 55 °C to dissolve).

3.2.2 Q-PCR for Relative mtDNA Copy Number

1. Analyze relative mtDNA copy number by quantifying mitochondrial cytochrome B (CytB) gene against nuclear amyloid precursor protein (APP) gene in an SYBR green quantitative PCR assay. The genes are amplified in separate reactions and results compared to a control reference sample.
2. Make Q-PCR reaction mix according to the manufacturer's instructions by mixing the primers (final concentration 500 nM for each primer) with the master mix (*see* **Note 9**).
3. Dilute all DNAs to a concentration of 5 ng/μl. Use 1–2 μl as a template for the mitochondrial CytB reaction and 5 μl from the same dilution as a template to the nuclear APP gene (*see* **Note 10**).
4. Run quantitative PCR according to your instruments normal protocol (*see* **Note 11**).
5. After the run remove any major outliers and calculate averages for the triplets. Use "delta delta Ct" method to calculate the relative mtDNA copy number. The power tells the relative copy number compared to the reference sample (*see* **Note 12**).
   (a) Delta Ct (dCt) = Ct (CytB) − Ct (APP).
   (b) Delta delta Ct (ddCt) = dCt (sample) − dCt (control).
   (c) Power = $2^{-\mathrm{ddCt}}$.

3.3 Analysis of Respiratory Chain Complexes

3.3.1 Whole Cell Lysates

1. Wash cells with PBS and collect in 1 ml of cold PBS using cell scraper (*see* **Note 13**).
2. Transfer to an Eppendorf tube and pellet by centrifugation at 1,000 × *g* for 5 min. Remove the supernatant.
3. Suspend the cell pellet in 100 μl of ice-cold lysis buffer (1.5 % DDM in PBS with freshly added protease inhibitors) (*see* **Note 14**).
4. Allow the tube to stand on ice for 30 min, vortex every 10 min.
5. Centrifuge 14,000 × *g* for 15 min at 4 °C, collect the supernatant (cell lysate), and discard the pellet (debris).
6. Measure protein concentration using Bradford assay or other suitable method.

3.3.2 Protein Concentration Measurement by Bradford Assay

1. Dilute Bradford Reagent 4:1 with water and run through a syringe filter.
2. Prepare standards from BSA stock (1 mg/ml; 0.8 mg/ml; 0.6 mg/ml; 0.4 mg/ml; 0.2 mg/ml; 0 mg/ml) (*see* **Note 15**).
3. Plate standards and samples in triplicate (10 μl per well) on a 96-well plate suitable for your plate reader.
4. Add 200 μl of diluted Bradford reagent to each well and let stand for 5 min.

5. Measure the absorbance with a plate reader with 595 nm filter according to your plate reader instructions.
6. Use the results first to graph the standard curve and then determine the unknown sample protein concentrations from the standard curve.

3.3.3 Western Blotting for Respiratory Chain Complexes

1. Add an equal volume of 2× Laemmli Sample Buffer to each sample (*see* **Note 16**) and load equal amounts of protein (10–20 μg of total protein from cell lysate) into the wells of the SDS-PAGE gel, along with molecular weight markers (*see* **Note 17**).
2. Run the gel for 1–2 h at 100 V. The time and voltage may require some optimization; we recommend following the manufacturer's instructions for your system.
3. Transfer the proteins to a PVDF or nitrocellulose membrane. Activate PVDF with methanol for 1 min and rinse with transfer buffer before preparing the stack. Transfer time and voltage may require optimization. We recommend following the manufacturer's instructions for your transfer system.
4. Block the membrane with 5 % milk in TBST for 1 h at RT or o/n at 4 °C.
5. Incubate membrane with appropriate dilutions of primary antibody in TBST with 1 % BSA overnight at 4 °C or for 2 h at room temperature (*see* **Note 18**).
6. Primary antibodies, their running sizes, which secondary antibody to use with them, and their dilutions:

   CI (39 kDa, mouse, 1:2,000).

   CII (70 kDa, mouse, 1:10,000).

   CIII (43 kDa, mouse, 1:2,500).

   CIV (39 kDa, boiled sample 57 kDa, mouse, 1:500).

   CV (53 kDa, mouse, 1:1,000).

   TOM20 (20 kDa, rabbit, 1:500).

   b-tubulin (55 kDa, rabbit, 1:5,000) used as a loading control.
7. Wash the membrane three times with TBST, 5 min each.
8. Incubate the membrane with 1:100,000 dilution of HPR-conjugated secondary antibody against mouse or rabbit IgG in 1 % BSA in TBST at room temperature for 1 h.
9. Wash the membrane three times with TBST, 5 min each.

10. Detect with chemiluminescence or colorimetric detection method according to the specific instructions of your system. We use Immun-Star Western C reagent and ChemiDoc XRS detection system (Bio-Rad).
11. Quantify the expression of the respiratory chain (RC) proteins against TOM20 and/or β-tubulin.

4 Notes

1. In order to maintain mtDNA heteroplasmy and high mutation loads in the fibroblasts, the primary cell lines should be established and cultured in the presence of uridine (200 μM) and pyruvate [10]. Due to the mitochondrial bottleneck and segregation of mtDNA during reprogramming, it is possible to derive iPS clones with high mutation levels even when the starting fibroblasts have low mutation loads.
2. To ensure for not selecting against mtDNA mutations during reprogramming, it is necessary to include uridine (200 μM) and pyruvate to the culture medium during all stages of cell culture [10]. The iPSCs are naturally glycolytic and need high glucose medium supplemented with pyruvate; however also subsequent differentiation of the iPSCs should be done in high glucose, pyruvate and uridine containing medium.
3. The cells should be in an exponential growth phase as high growth rate of the starting fibroblasts is critical for good reprogramming efficiency.
4. Antibiotics are optional in all culture medium; however, immediately after electroporation, it is best to culture the cells overnight without antibiotics, to allow them to recover from the electroporation without any additional stress.
5. For electroporation we use Neon electroporation system (Invitrogen), but other systems should work as well. The method is explained for Neon transfection system with 100 μl tips, but also 10 μl tips can be used.
6. The plating density depends on the growth rate of the fibroblasts. If the cells grow very fast, lower densities may be useful to prevent overgrowth of the cells during subsequent culture.
7. Even though the surface size of a 24-well dish is the same as a 4-well dish, we find it easier to use 4-well dishes, as these have lower edges, which will make the mechanical cutting easier.
8. During initial passaging, some differentiation and cell death may occur. This is normal, and mechanical cutting will help to select for the good pluripotent cells.

9. Q-PCR reactions should always be done in triplicates in order to identify possible pipetting errors.
10. Less template is needed for the CytB reaction since there are multiple mtDNA copies in a cell versus a single nuclear genome. However, in order to avoid mistakes due to differences in template concentrations, it is crucial that the same dilution is used for both reactions.
11. PCR protocol can be adjusted according to the instrument in use. We use a 2-step protocol, with melting temperature at 60 °C and 40 cycles.
12. Choose one sample as the reference sample to which other samples are compared. The relative expression of the reference sample will be 1.
13. All the subsequent protein extraction steps should be done on ice and with ice-cold buffers.
14. The exact buffer volume depends on the number of cells. Use 100 μl of buffer for $1–5 \times 10^6$ cells.
15. The standard curve can be adjusted to cover the concentration range the samples are in. Alternatively the samples may be diluted, so that they are in the range that the standard curve covers.
16. For the RC complex proteins, it is not necessary to boil the samples.
17. Homemade or precast gels can be used for running the protein samples. In general we use 4–20 % precast TGX gradient gels (Bio-Rad). Using gradient gels allows good separation of proteins with different sizes (size range for the mitochondrial proteins 20–70 kD) and several proteins can be detected from a single membrane. Two membranes are needed to detect all respiratory chain complexes and loading controls.
18. In our hands the β-tubulin antibody works best when incubated overnight at 4 °C; all other antibodies work well in both conditions. Instead of the β-tubulin, any other house-keeping protein can also be used as a loading control.

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Skin Biopsy and Patient-Specific Stem Cell Lines

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Abstract

The generation of patient-specific induced pluripotent stem (iPS) cells permits the development of next-generation patient-specific systems biology models reflecting personalized genomics profiles to better understand pathophysiology. In this chapter, we describe how to create a patient-specific iPS cell line. There are three major steps: (1) performing a skin biopsy procedure on the patient; (2) extracting human fibroblast cells from the skin biopsy tissue; and (3) reprogramming patient-specific fibroblast cells into the pluripotent stem cell stage.

Keywords: Human iPS, Skin biopsy, Fibroblast, Reprogramming, Sendai virus

1 Introduction

Induced pluripotent stem (iPS) cells reprogrammed from somatic cells have allowed for the generation of patient-specific disease cells, which carry disease phenotype in vitro. Interest in generating human-induced pluripotent stem (hiPS) cells for stem cell based disease modeling has overtaken that of patient-specific human embryonic stem (ES) cells due to the ethical, technical, and political concerns associated with the latter (1). Patient-specific iPS cells have potential in human disease modeling and regenerative medicine. The in vitro phenotype of disease-specific iPS-derived cells can be used to bridge the knowledge gap between the clinical phenotype and molecular or cellular pathophysiology, along with further applications, such as creating new strategies for drug screening or developing novel therapeutic agents. By using patient-specific hiPS cells, we can prove that a disease is caused by a novel gene mutation and hypothesize potential treatment options before moving to more expensive animal studies (1). The hiPS cell-based disease models may also assist in the development of novel treatments for clinical trials (2, 3).

The generation of human iPS cell lines has already been widely described since Takahashi's and Yamanaka's report on

obtaining pluripotent cells from somatic cells (4). At the time, the scientific community's primary concern regarding the limitation of Takahashi and Yamanaka's technique was that the integration of retroviral-mediated transduction into the host genome includes the risk of insertional mutagenesis (5). In our protocol, we use the Sendai virus (6, 7), a footprint-free RNA virus that carries no risk of altering the host genome (8), to generate patient-specific iPS cell lines.

Successfully establishing a patient-specific iPS cell line requires three steps: (1) obtaining human subject committee approval and performing a skin biopsy on patient; (2) deriving human fibroblast cells from the skin biopsy tissue; and (3) reprogramming patient-specific fibroblast cells into the pluripotent stem cell stage. Here, we summarize the materials and methods necessary for the entire procedure.

2 Materials

All the reagents, medium, components, and tools are sterile.

2.1 Basic Supplies

1. Laminar flow hood.
2. Water bath, 37 °C.
3. Pipet-Aid.
4. Plastic serological pipets: 1, 2, 5, and 10 ml.
5. Incubator, 5 % CO_2.
6. Tabletop centrifuge.
7. Spray bottle, 70 % ethanol.

2.2 Materials for Skin Biopsy Procedure

1. Biopsy collect medium: RPMI1640, 1× Anti-Anti.
2. Disposable biopsy punches, diameter 4 mm.
3. Xylocaine (lidocaine HCL and epinephrine injection, USP).
4. Sterile fenestrated towel.
5. Povidone–iodine, USP swab stick.
6. Sterile, powder-free surgical gloves.
7. 5-0 VICRYL coated suture.
8. 3/10 ml insulin syringe.
9. Sterile gauze pad.
10. Autoclaved forceps, needle holder, scissors.
11. 15 ml conical tube.
12. Band-Aid.

13. 70 % ethanol wipes.

2.3 Materials for Preparation of Human Fibroblast Cultures from Skin Biopsy Tissue

1. 6-well cell culture plate.
2. Plastic serological pipets.
3. Biopsy plating medium: DMEM, 10 % fetal bovine serum (FBS), 1× nucleosides, 1× L-glutamine, 1× nonessential amino acids, 1× Anti-Anti, 0.1 mM β-mercaptoethanol.
4. Microscope coverslips.
5. Sterile micro-forceps and scissors.
6. Sterile scalpel.
7. Silicone grease.
8. Phosphate buffered saline (PBS).
9. 0.05 % trypsin–EDTA.
10. Fibroblast culture medium: DMEM, 10 % FBS, 1× L-glutamine, 1× sodium pyruvate, 1× penicillin–streptomycin.
11. 10 cm petri dish.
12. 0.5 % gelatin coating solution (in water).

2.4 Materials of Human iPS Cells Generation

1. KO-DMEM medium: KnockOut DMEM, 15 % KnockOut serum replacement, 1× L-glutamine, 1× nonessential amino acids, 1× penicillin–streptomycin, 0.1 mM β-Mercaptoethanol, basic fibroblast growth factor (bFGF) 10 ng/ml.
2. Fibroblast medium: DMEM (high glucose), 10 % FBS, 1× L-glutamine, 1× penicillin–streptomycin, 1× sodium pyruvate.
3. 12-well cell culture plate.
4. Y-27632 (ROCK inhibitor).
5. 0.1 % gelatin solution.
6. Freezing solution for ES cells.
7. TrypLE Express (1×).
8. Phosphate buffered saline (PBS).
9. Mitomycin-C treated mouse embryonic fibroblast (MEFs, feeders).
10. CytoTune-iPS reprogramming Kit (Life technologies, A13780).
11. 4-well cell culture plate.

1. Matrigel matrix.
2. 1 ml insulin syringe.
3. Hypodermic disposable needle, 18G.
4. Ice and ice bucket.

2.5 Materials for Characterization Assay

5. SCID mice.

2.5.1 Materials for Teratoma Assays

2.5.2 Materials and Markers for Immunofluorescent Assay

Materials for immunofluorescent assay have been widely available and primary antibodies of pluripotent markers are: OCT4 Santa Cruz sc-9081 Rabbit poly, SOX2 R&D Systems 245610 Mouse IgG, TRA-1-60 Millipore (Chemicon) MAB4381, Mouse, IgM, SSEA4 Millipore (Chemicon) MAB4304 Mouse IgG, Nanog R&D Systems AF1997 Goat poly.

3 Methods

All procedures excluding the skin biopsy surgery are preformed in the laminar flow hood.

3.1 Skin Biopsy Procedure

Here (*see* **Note 1**), we briefly describe this procedure. It should be done by a dermatologist or an appropriately trained physician.

1. The biopsy must be performed under sterile conditions. Place a sterile fenestrated towel over the biopsy area. The biopsy site will be at the center of the fenestrated location.
2. Anesthetize the skin locally by subcutaneous injection of 1 % lidocaine.
3. The site of biopsy should be disinfected with 5 % povidone–iodine twice. For disinfection, use a povidone–iodine swab to wipe the site of biopsy beginning from the center in circular movements and proceeding to the outer edges. Air-dry. Take 15 s for this procedure and repeat it using a new swab.
4. Skin biopsy is performed with a sterile 4 mm skin punch and cut off by a sterile scissors. Transfer the skin biopsy with sterile forceps into a sterile 15 ml plastic tube containing 10 ml biopsy collect medium. Place the tube under room temperature.
5. Suture the edges of the wound with 6.0 VICRYL coated dissolvable suture and bury the knots.
6. Clean the sutured area with sterile 70 % ethanol wipes and then place a Band-Aid to cover the suturing site.

3.2 Fibroblast Culture and Collection from Skin Biopsy Tissue

The entire procedure usually takes about 4 weeks: 2 or 3 weeks for fibroblasts to expand to cover most of the area underneath the coverslips, then another week to collect the fibroblasts from the plating dish and passage them to cover a 10 cm culture dish.

1. Rinse the biopsy once in PBS or collection medium.

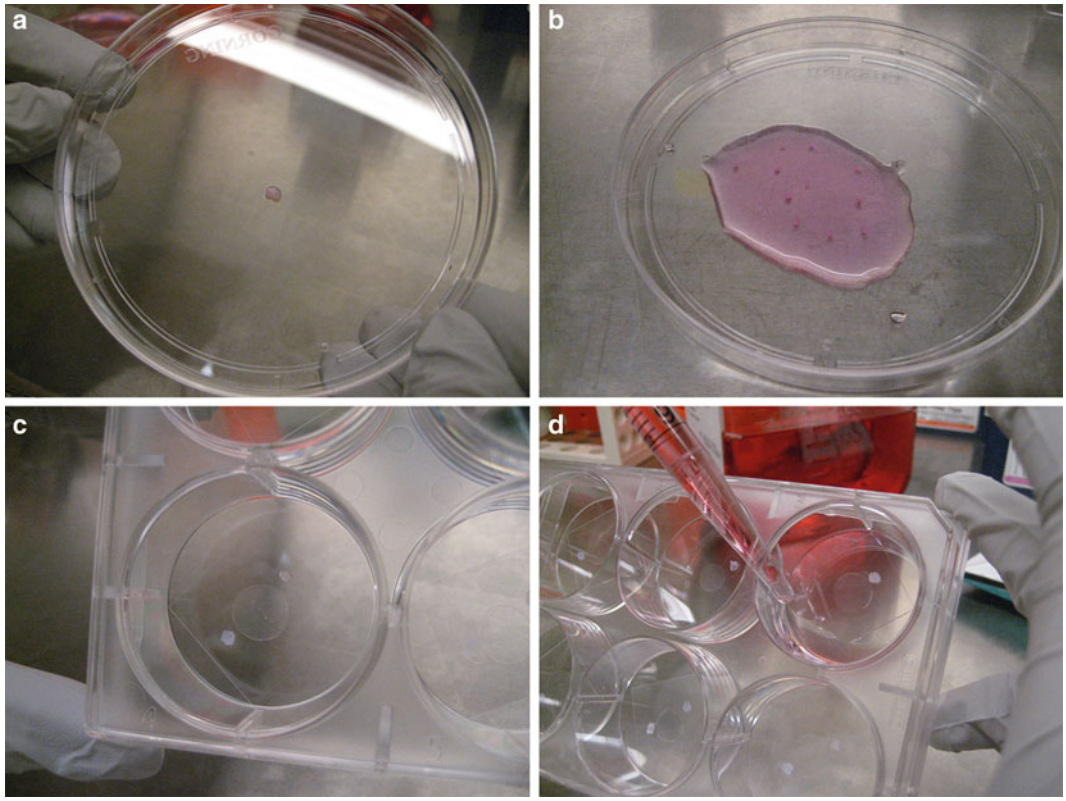

Fig. 1 Key steps of plating human skin biopsy sample on culture dish. (**a**) Place biopsy tissue in a 10 cm petri dish. (**b**) Mince the biopsy core into 10–12 pieces. (**c**) Take a coverslip and place over the grease and minced biopsy pieces. (**d**) Tilt the dish slightly. Slowly add biopsy plating media into the dish

3.2.1 Preparation of Human Fibroblast Cultures from Skin Biopsies

2. Place biopsy tissue in a 10 cm petri dish with a little bit of medium (Fig. 1a).
3. Carefully hold the biopsy punch with sterile forceps and remove the scarfskin (*see* **Notes 2** and **3**), then carefully mince the biopsy core into 10–12 pieces (Fig. 1b).
4. Using a sterile 1,000 μl pipet tip, transfer autoclaved silicone grease and place one drop in the center of each well in a 6-well culture plate.
5. Using sterile forceps, place two to three pieces of minced biopsy around the silicon grease drop. Plate all tissue.
6. Take a coverslip and place over the grease and minced biopsy pieces. Press on coverslip (Fig. 1c) (*see* **Note 4**).
7. Tilt the dish slightly. Slowly add 5 ml of biopsy plating medium. Be careful to not lift off the cover slip (Fig. 1d). The air below the cover slip should be displaced by medium. Gently apply pressure on each cover slip to ensure that it is well attached. Place into incubator. Do not disturb the cultures for at least 4 days.
8. Change medium every 5–7 days.

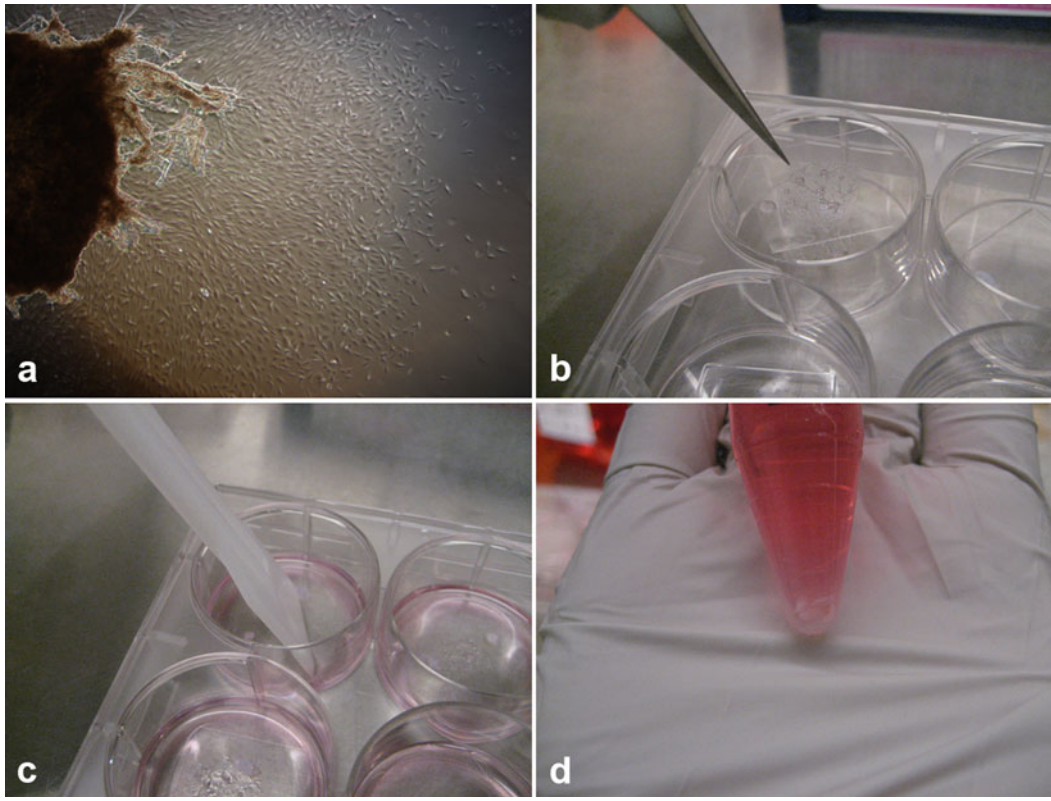

Fig. 2 Key steps of collecting fibroblast cells from biopsy plating dish. (**a**) Fibroblasts cells growing out from skin tissue. Fourteen days post plating. (**b**) Using a sterile forceps, carefully peel off the coverslip from the bottom of each well and overturn inside the well. (**c**) After digestion, harvest the cells by a sterile scalpel. (**d**) After resuspending cell pellet and tissue, wait the tissue to settle to the bottom of the conical tube

3.2.2 Collection of Fibroblast Cells from Biopsy Plating Dish

1. Cultures are ready to be split when the entire coverslip is covered with fibroblasts (in 2–3 weeks). Fibroblasts grow on the glass as well as on the plastic (Fig. 2a).
2. Pre-warm trypsin, prior to use. Gelatinize 6-well culture plate for every 6-well biopsy plating plate.
3. Remove topic medium and wash the plate surface with PBS. With a sterile forceps, carefully peel off the coverslip from the bottom of each well and overturn inside the well. The cell growth surface should be facing up (Fig. 2b). Add 1 ml of pre-warmed trypsin, ensuring the overturn coverslip is covered with trypsin, and replace into incubator for 5 min.
4. Check trypsin digestion after 5 min. When the cells are no longer attached to the coverslip, harvest the cells and biopsy tissue together by a sterile scalpel, and then transfer them into a 15 ml conical tube (Fig. 2c). Inactivate trypsin with 1:1 volume of fibroblast culture medium.

5. Spin down at 500 × *g* for 4 min. Aspirate supernatant and resuspend the cell pellet and tissue with fibroblast culture medium. Wait 30 s to allow the tissue to settle to the bottom of the conical tube (Fig. 2d).
6. Remove gelatin from pre-coated 6-well plate and transfer cell suspension to each well (discard the tissue).
7. Change medium every 1 or 2 days for regular culture until the cell grow to confluency for the next passage.

3.3 iPS Generation Protocol with Sendai Virus

1. Plate 5×10^4 fibroblast cells (*see* **Note 5**) in each well of a 12-well plate one day ahead the transfection day.
2. Culture fibroblast cells in an incubator (37 °C, 5 % CO_2) overnight to make sure that the cells extend and adhere to the dish.
3. Take out the Sendai viruses (*see* **Note 6**) expressing the four Yamanaka factors (*OCT3/4*, *SOX2*, *KLF4*, and c-*MYC*) from stock (CytoTune-iPS reprogramming, Life Technologies, USA) at −80 °C and thaw them following manufacturer instruction.
4. Calculate volumes of each virus used for one well of cells (5×10^4 cells per well) at a multiplicity of infection (MOI) of 3.
5. Aliquot the appropriate volume of each virus for every 5×10^4 cells, as decided in **step 4**, to 500 μl fibroblast culture medium (every 500 μl virus–medium mixture contains the four Yamanaka factors for one well of cells).
6. Remove the culture medium completely from the cells prepared in **step 1**. For every 5×10^4 cells (each well) apply 500 μl virus–medium mixture gently to each well. Swirl the plate slightly to make the mixture covers the entire cell layer.
7. Place the plate into an incubator (37 °C, 5 % CO_2) overnight.
8. The next day, add another 500 μl of fibroblast culture medium to each well. Place the plate into incubator (37 °C, 5 % CO_2) overnight.
9. On the following day, remove the virus-containing medium and replace with KO-DMEM medium. Continue incubation (37 °C, 5 % CO_2) for an additional 6–7 days, changing the medium every day with KO-DMEM medium.
10. One day before the day of cell passage in **step 8**, prepare a feeder cell-coated plate by inoculating Mitomycin-C treated MEF cells on gelatin-coated cells. To coat cells with gelatin, add 2 ml of 0.1 % gelatin solution per well of a 6-well, swirl to cover the entire surface with the solution, and let stand at 37 °C for 30 min. Remove the gelatin solution immediately before plating. MEF cells should be plated in 6-well plates at 2×10^5 cells per well. On the following day, change the medium with fibroblast culture medium.

11. 7–8 days after Sendai transduction, remove the medium, wash the cells once with PBS, add 500 μl per well of TrypLE express and let it incubate at 37 °C for 4 min. After 4 min, take the plate out of the incubator, remove the TrypLE express carefully and leaves the half-detached cells in the wells. Apply 2 ml KO-DMEM medium containing 10 μM ROCK inhibitor in each well and resuspend the cells by gently pipette up and down. Chunks of cells may remain in this step. Transfer cells onto the feeder plate. Cells from one well of a 12-well plate should be transferred to one well of 6-well feeder plate.
12. Return the culture plates to the incubator (37 °C, 5 % CO_2). After 24 h, change the medium with KO-DMEM medium (without ROCK inhibitor). Change medium every day with freshly prepared KO-DMEM medium.

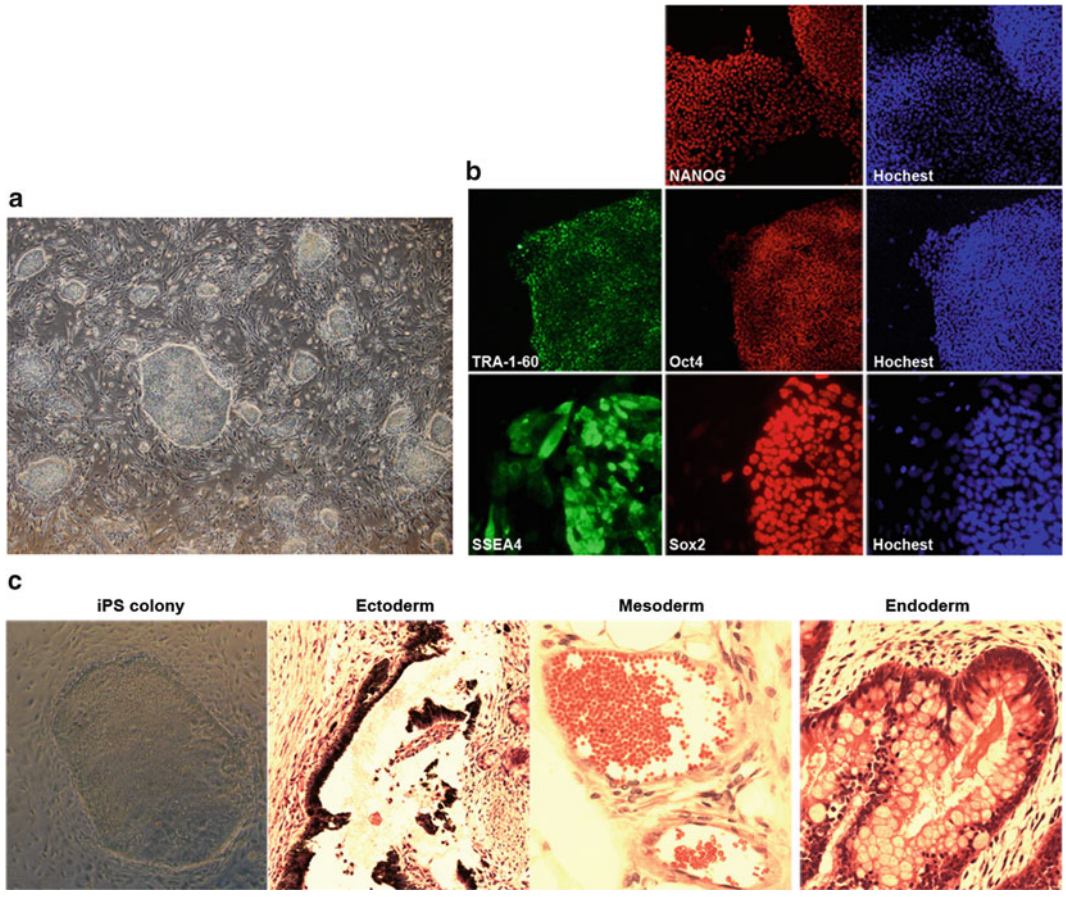

Fig. 3 Generation and characterization of human iPS cells. (**a**) iPS cell colonies start to appear on infection plate, 20 days post infection. (**b**) Anticipated results of iPS Characterization assay: immunofluorescent assay, human iPS cells express surface markers of human ES cells. (**c**) Anticipated results of iPS Characterization assay: teratoma assay, teratoma generated from human iPS cells containing tissues derived from three primary germ layers

13. Colonies should be observed 6–7 days after passage (Fig. 3a). One day before passaging colonies, prepare feeder cells by inoculating MEF cells at 4×10^4 cells per well (4-well plate). The wells should be pre-coated with gelatin.
14. Apply 750 µl pre-warmed 10 µM ROCK inhibitor contained KO-DMEM medium to each well of 4-well plate right before use.
15. Microdissect each iPS colony into chunks of about 100–150 cells using sterile glass hooks under microscope. The hook is used to gently split apart pieces of the colony. Cut a grid into the colony with the back of the hook to pull the pieces away from the colony. The size of each division should be sufficiently large to survive the cutting and adhering to the feeder layer (*see* **Note 7**).
16. Transfer four to five colony chunks into one well of a 4-well plate, prepared in **step 12**, using 200 µl micropipets.
17. Replace the 4-well plate to the incubator (37 °C, 5 % CO_2).
18. On the next day, change the medium with KO-DMEM medium. Change medium daily with the KO-DMEM medium.
19. Passage cells 1 week after the colony transfer in **step 15** using conventional methods for iPS cell cultures (9) (*see* **Note 8**).

3.4 iPS Characterization Assay

There are two commonly used assays for iPS cells. Immunocytochemistry assays are established means for scoring stem cell pluripotency (Fig. 3b). The ability for iPS cells to generate teratomas in immune-deficient mice is used to assess their capabilities to differentiate into the three primary germ layers (Fig. 3c).

3.4.1 Immunofluorescent Assay

Protocols for immunofluorescence are widely available. Here we describe briefly this application for cells in culture on coverslip plates, clear-bottom dishes for brief imaging (Fig. 3b).

1. Wash plates once in 1× PBS.
2. Fix in about 2 ml of 4 % PFA for 20 min at room temperature (RT).
3. Wash two times in 1× PBS. The dishes can be stored at 4 °C if sealed with Parafilm.
4. Block with 3 % normal serum (species dictated by host of secondary antibody, usually Donkey or Goat for Alexa conjugated secondaries) with 0.1 % Triton X in 1× PBS (minus) for 30 min at RT.
5. Remove block and add primary antibodies at 1:100 mentioned in Section 2.5.2.
6. Incubate at 4 °C overnight.
7. Wash three times, with at least 3 min per wash, in PBST (PBS with 0.1 % Tween 20) at RT.
8. Add secondary diluted in Block.

9. Incubate at RT for 30 min or overnight at 4 °C.
10. Wash twice in PBST for each 3 min wash.
11. If counterstaining with SytoxOrange (or other nuclear counterstain), incubate in PBST. SytoxOrange is diluted at 1:25,000.
12. Wash twice in PBST and leave in PBST. They can be stored at 4 °C sealed with Parafilm or imaged. If photobleaching is a problem, the PBST can be removed and a drop of VECTASHIELD mounting medium added. A coverslip can be placed on top to prevent evaporation.

3.4.2 Teratoma Assay

1. iPS cells are harvested as described in previous procedures for passaging and suspension in medium (250 μl per injection), mixed with an equal volume of thawed Matrigel and transferred to cold cryotubes. The mixture is held on ice until loaded into the syringe just before injecting.
2. The iPS cells are loaded into syringes fitted with an 18G needle. Load the cells into the syringe by drawing in a small amount of medium followed by the iPS cell suspension before attaching the needle.
3. The suspension is injected subcutaneously, targeting the needle beneath the skin on the rear flank of the SCID mouse.
4. Monitor the mice and their sites of injection weekly for 6–22 weeks. The mice should be weighed weekly and watched for signs of infection during the incubation period.
5. Teratomas can be recovered by dissection with surrounding tissue and usually arise between 6 and 8 weeks after grafting. They are fixed in formalin and sent for histological examination by a pathologist.

4 Notes

1. We described briefly the procedure for obtaining a skin biopsy from a patient. A dermatologist or other appropriately trained physician should perform skin biopsy under human subjects ethical guidelines.
2. Rinse with medium when cutting the skin tissue into pieces, in case the small skin piece dries out.
3. Remove the epidermis to avoid growth of keratinocytes.
4. After putting coverslips on the top of the silicone gel and skin tissue, use pipets to press the coverslip to attach it well. There should be barely any space between the coverslip and the culture dish.

5. Use cells with as early passage number if possible since the passage number may affect the efficiency of reprogramming.
6. The iPS cells generated by the use of Sendai do not retain any vector DNA sequences.
7. Concerning the size of iPS chunks during iPS colony picking, if a piece is too large, it will tend to form an embryoid body-like structure on the feeder layer and it will take too long for the entirety of a large colony to come into contact with the feeders. The resulting colony will have an area of differentiation in the center arising from the embryoid body-like structure.
8. Complete growth medium is exchanged on the growing colonies every day as the feeder layer can use up nutrients quickly. The cell cycle for this line is about 24–36 h. The lines should culture for no more than 6 days to a week.

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Directed Myogenic Differentiation of Human Induced Pluripotent Stem Cells

Emi Shoji, Knut Woltjen, and Hidetoshi Sakurai

Abstract

Patient-derived induced pluripotent stem cells (iPSCs) have opened the door to recreating pathological conditions in vitro using differentiation into diseased cells corresponding to each target tissue. Yet for muscular diseases, a method for reproducible and efficient myogenic differentiation from human iPSCs is required for in vitro modeling. Here, we introduce a myogenic differentiation protocol mediated by inducible transcription factor expression that reproducibly and efficiently drives human iPSCs into myocytes. Delivering a tetracycline-inducible, *myogenic differentiation 1* (*MYOD1*) *piggyBac* (PB) vector to human iPSCs enables the derivation of iPSCs that undergo uniform myogenic differentiation in a short period of time. This differentiation protocol yields a homogenous skeletal muscle cell population, reproducibly reaching efficiencies as high as 70–90 %. *MYOD1*-induced myocytes demonstrate characteristics of mature myocytes such as cell fusion and cell twitching in response to electric stimulation within 14 days of differentiation. This differentiation protocol can be applied widely in various types of patient-derived human iPSCs and has great prospects in disease modeling particularly with inherited diseases that require studies of early pathogenesis and drug screening.

Keywords: Skeletal muscle cell, Human iPS cells, Myogenic differentiation, MyoD, *piggyBac* vector, GFP, Doxycycline inducible differentiation

1 Introduction

Most muscular diseases do not have curative treatment and are limited to palliative care that fails to prevent progression. Additionally, for many myopathies, the pathology remains unclear, although the causative genes have been identified. Thus, there is a requirement to investigate the precise pathology of each myopathy. Human induced pluripotent stem cell (hiPSC) technology has enabled patient-specific models of human disease in vitro [1, 2], since hiPSCs offer indefinite proliferation and multilineage differentiation capacity [3]. Establishing methodology for versatile and practical myogenic differentiation is of particular importance for skeletal muscle cell research, and the identification of therapeutic approaches to overcome inherited muscle diseases. Methods for the

differentiation of hiPSCs to skeletal muscle cells have taken several approaches, lending from our knowledge of in vivo development [4–6]. *PAX7*, a paired box transcription factor that maintains the adult satellite cell [7], and *MYOD1*, a master transcription factor of skeletal muscle cell differentiation and essential gene in skeletal muscle cell lineage and muscle stem cell function in adult skeletal muscle [8], have both been conditionally expressed in human ES and iPS to induce directed differentiation and obtain large quantities of myogenic precursors [9].

Direct conversion of fibroblasts to muscle cells by *MYOD1* was originally demonstrated in 1987 [10]. Reports of skeletal muscle cell differentiation from hiPSC-derived fibroblasts [11] or hiPSC-derived mesodermal cells [12] demonstrate the superior potential of *MYOD1* for myogenic differentiation. Yet, a universal method effective at delivering MyoD to various hiPSC lines was only recently reported [13]. A *piggyBac* (PB) transposon vector [14] carrying doxycycline (dox)-inducible *MYOD1* (Fig. 1) can be delivered directly into hiPSCs (Tet-MyoD-hiPSCs), excluding the redundant derivation of fibroblasts or mesoderm prior to myogenic differentiation. Moreover, optimized 2D culture conditions promote consistent differentiation through uniform contact of single cells with media and growth factors. Using this approach, mature myocyte differentiation from Tet-MyoD-hiPSCs can be achieved within 2 weeks, and with high efficiency [13]. Myocytes obtained from Tet-MyoD-hiPSCs express skeletal muscle-specific proteins such as Dystrophin, which is only detected in mature skeletal muscle cells. Functionality and maturity of differentiated myocytes are indicated by cell fusion and cell twitching in response to electric stimulation. Moreover, through the reprogramming process from the patient's fibroblasts, intact matured myocytes without being affected from external factors, such as inflammation or any other signaling molecules, can be produced from Tet-MyoD-hiPSCs.

Our PB-delivered *MYOD1* approach has successfully induced myocytes differentiation from Miyoshi myopathy patient-derived hiPSCs [13], and is proving invaluable in the derivation of disease models from various myopathic patient hiPSCs (Sakurai, *unpublished data*). In each case, the delivery of dox-inducible *MYOD1* by the PB transposon offered stable and efficient skeletal muscle cell differentiation. Herein, we describe the steps required to derive Tet-MyoD-hiPSCs: from PB transfection, through *MYOD1* induction and culture adaptation, and finally the evaluation of differentiated myocytes. This straightforward and tractable technology has great prospects in muscle cell research towards development of new drugs for muscular diseases.

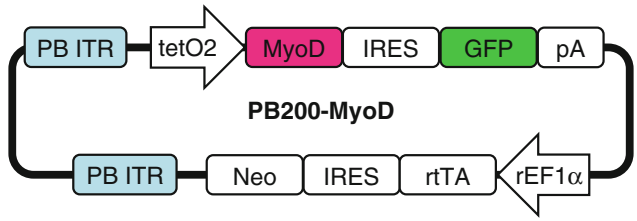

Fig. 1 The dox-inducible *MYOD1*-expressing *piggyBac* vector, PB200-MyoD, derived from PB-TAG-ERN

2 Materials

2.1 Human iPS Cell Culture

1. 201B7 hiPSCs, established from human dermal fibroblasts by retroviral overexpression of the four Yamanaka factors (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*) as previously described [3]. Following the suggested optimization steps, this differentiation protocol should be applicable to all hiPSC lines.
2. Primate ES cell medium (ReproCELL, RCHEMD001) supplemented with 10 ng/mL recombinant human basic fibroblast growth factor (bFGF: Wako, 064-04541).
3. Feeder cells inactivated with mitomycin-C. A feeder line such as SNL (a subclone of the STO mouse fibroblast cell line) that is resistant to neomycin is required to survive drug selection.

2.2 PB200-MyoD Transfection with FuGENE® HD to hiPSCs

1. Plasmids: PB200-MyoD (Fig. 1) and pCAG-PBase (*piggyBac* transposase). The PB200-MyoD vector is available by MTA upon request to the authors. The parental PB Gateway® Destination vector PB-TAG-ERN (RDB13244) and pCAG-PBase (RDB13241) [15] are available through the RIKEN BRC DNA Bank (dna.brc.riken.jp).
2. FuGENE® HD (Roche, 4709705).
3. OPTI-MEM (GIBCO, 31985).
4. CTK solution: To prepare 50 mL of CTK solution, mix 5 mL of 2.5 % Trypsin (Life technologies, 15090-046), 5 mL of 1 mg/mL collagenase-IV (Life technologies, 17104-019), 500 μL of filtered (0.2 μm) 0.1 M $CaCl_2$, and 10 mL of Knockout Serum Replacement (KSR: Life technologies, 10828-028) in 30 mL of sterile deionized water.
5. Neomycin sulfate (Neo: Nacalai Tesque, 146-08871).
6. 5 mL Polystyrene FACS tubes (BD Falcon™, 352058).

2.3 Differentiation to Skeletal Muscle Cells

1. Primate ES cell medium (ReproCELL, RCHEMD001).
2. 5 % KSR/αMEM: To prepare 50 mL of 5 % KSR/αMEM, add 2.5 mL of KSR and 100 μL of 100 mM 2-Mercaptoethanol (2-ME: Nacalai Tesque, 2143882) to 47.5 mL of penicillin/streptomycin supplemented αMEM medium.

3. 2 % horse serum/DMEM: Prepare 50 mL of 2 % horse serum/DMEM medium by adding 1 mL of horse serum (Sigma, H1138) and 100 mM 2-ME to DMEM basal media. Prepare DMEM basal media by adding 1 % of L-Glutamine and 2.5 mL of penicillin/streptomycin (Nacalai Tesque, 2625384) to 500 mL of DMEM high glucose (GIBCO, 11960069). To complete 2 % horse serum media, add recombinant Human IGF-I (Peprotech, 100-11) before medium change.
4. Matrigel- (Corning, 356231) or collagen-I-coated 6-well plate (AGC, 4810-010).
5. Neomycin sulfate (Neo: Nacalai Tesque, 146-08871).
6. Doxycycline hyclate (Dox: LKT Labs, D5897).
7. Y-27632, ROCK inhibitor (Nacalai Tesque, 08945-84).
8. 0.25 % Trypsin/1 mM EDTA (Nacalai Tesque, 3555464).
9. CTK Solution.

3 Methods

3.1 Transfection of PB200-MyoD into hiPSCs

Transfection of PB200-MyoD into hiPSCs may be carried out by either chemical transfection or electroporation. Here, we describe PB200-MyoD transfection using FuGENE® HD transfection reagent (Roche) and outline a simple approach to optimize hiPSC transfection conditions. For further details, refer to the manufacturer's protocol for FuGENE® HD. For a detailed protocol on PB electroporation into hiPSCs, refer to Kim et al. [15].

1. Prepare hiPSCs at approximately 60 % confluency on a 6-well plate with inactivated SNL feeder cells, in primate ES cell medium supplemented with 4 ng/mL bFGF.
2. Change media to primate ES cell medium supplemented with 10 ng/mL of bFGF just before the transfection.
3. Prepare a master mix for 7 wells by mixing 700 μL of OPTI-MEM and 7.0 μg each of PB200-MyoD and pCAG-PBase plasmids. Mix the solution thoroughly by pipetting and dispense 100 μL aliquots (total 2.0 μg of plasmid DNA) into each tube.
4. Prepare a concentration series of FuGENE® HD (ranging from 3.0 to 8.0 μL) to each tube (Fig. 2). Optimize transfection parameters by adjusting the concentration of transfection reagent and amount of plasmids for each hiPSC line. For most hiPSCs, a 1:3 ratio is optimum (*see* **Note 1**).
5. Mix each tube by vortexing for 1–2 s.
6. Incubate the tubes for 15 min at room temperature.

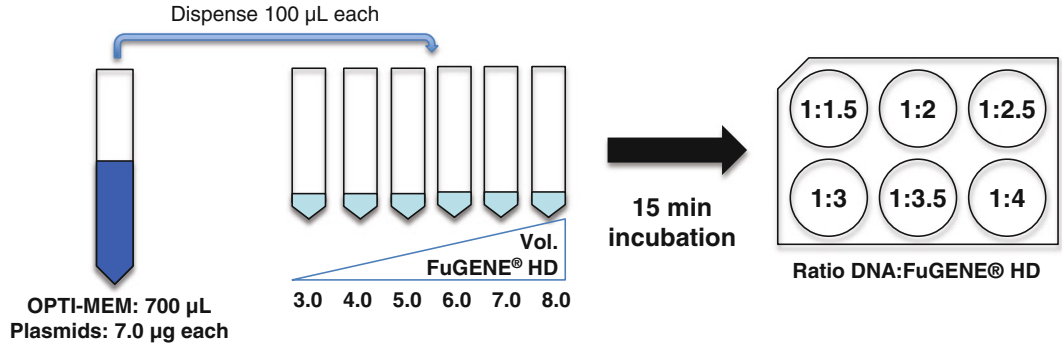

Fig. 2 Schematic outline for PB200-MyoD transfection. A concentration range is used to test optimal DNA: FuGENE® HD ratios for each hiPSC line

7. Add each mixture to the indicated well, dropwise (Fig. 2). Swirl to mix.
8. On the following day (~24 h), change media to 10 ng/mL bFGF added primate ES cell medium.
9. Two days after transfection, change media to primate ES cell medium supplemented with 100 µg/mL Neo and bFGF (*see* **Note 2**).
10. Maintain Neo selective pressure until colonies emerge (~4–8 days, depending on the hiPSC line and transfection efficiency).
11. At this stage, the cells can be passaged as a population or picked as individual clones.
12. Prepare a dish to test the induction level of MyoD indirectly by GFP in FACS, and directly by MyoD Western blot.
13. Prepare frozen stocks of Tet-MyoD-hiPSCs with appropriate expression.
14. Keep the passage number low, and maintain the cells in Neo until differentiation.

3.2 Skeletal Muscle Cell Differentiation

hiPSCs form tightly packed colonies demonstrating one of the characteristic features of pluripotent stem cells (Fig. 3a). In the absence of dox, Tet-MyoD-hiPSCs that emerged after at least 4 days of Neo drug selection still retain morphological and molecular properties of pluripotency (Fig. 3b, and data not shown). These cells are maintained in a pluripotent state until the addition of dox, which initiates uniform myogenic differentiation.

The dox-induced skeletal muscle cell differentiation consists of three phases: induction, differentiation, and maturation (Fig. 4). Skeletal muscle cell maturation is promoted in 2 % horse serum medium [16]. Administration of IGF-I also boosts maturation and enlarges the size of myocytes [17, 18]. Cell density is a critical parameter for efficient differentiation and should be tested empirically using the parameters suggested (*see* **Note 3**).

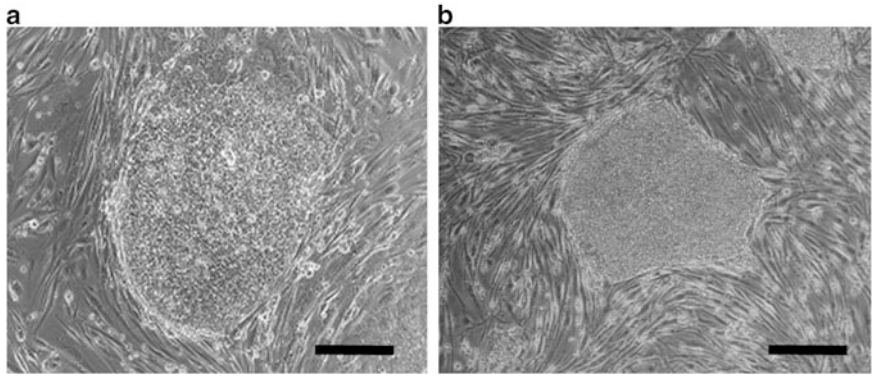

Fig. 3 Morphology of pluripotent parental and Tet-MyoD-hiPSCs. (**a**) hiPSCs generated by four-transcription factor reprogramming form colonies with characteristic morphology. (**b**) In the absence of dox, Tet-MyoD-hiPSCs retain a similar morphology. Scale bars: 200 μm

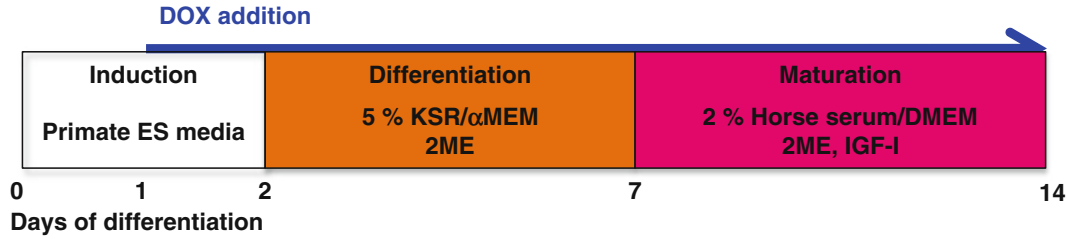

Fig. 4 Overview of the mature myocyte differentiation procedure. Dox addition initiates myocyte differentiation by overexpressing *MYOD1*. Differentiation process consists of induction, differentiation, and maturation phases

Dox-treated Tet-MyoD-hiPSCs take on a rounded cell structure and then begin to change to spindle-like structure that is characteristic of skeletal muscle cells (Fig. 5a). Furthermore, successfully differentiated myocytes will undergo cell fusion to form multinucleated myocytes (Fig. 5a, b). Myosin heavy chain (MHC), skeletal muscle actin (SMA), and creatine kinase, muscle type (CKM) are skeletal muscle-specific markers and can be detected in differentiated myocytes at day 9 of differentiation (Fig. 5b), and are used to gauge the efficiency of directed differentiation.

1. Prepare matrigel- or collagen-I-coated 6-well plates. For matrigel coating, dilute matrigel 1:50 in primate ES cell media and apply to the plate at least 2 h prior to starting the differentiation process.
2. To eliminate SNL feeder cells, wash plated cells with PBS and add 1 mL of CTK solution (for 100 mm dish) to the plated cells. Incubate at room temperature for 2–3 min and dislodge feeder cells by washing twice with PBS.

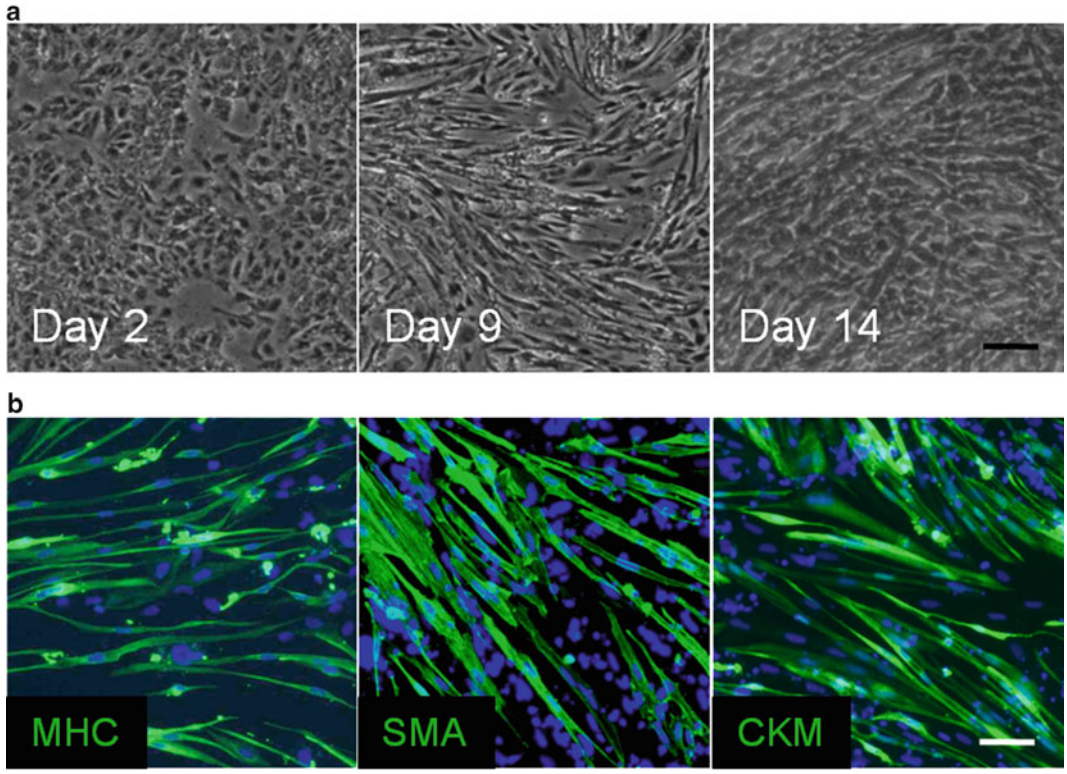

Fig. 5 Morphological changes through the differentiation process. (**a**) Rounded cells are observed at day 2 of differentiation and start to form spindle-like structures from day 4 of differentiation. By day 9 of differentiation, myocytes begin to fuse to form multinucleated skeletal muscle cells. Networks of myocytes are observed at day 14 that contract in response to electric stimulation. (**b**) Immunohistochemistry at day 9 of differentiation also confirms the maturation stage of differentiated myocytes. MHC, SKM, and CKM are skeletal muscle-specific markers. Scale bars: 50 μm

3. Detach and dissociate Tet-MyoD-hiPS colonies by adding 1 mL of 0.25 % trypsin and incubating the plate at 37 °C for 5 min.
4. Add at least an equal volume of primate ES cell media to neutralize the trypsin and dissociate the culture to a single-cell suspension by pipetting thoroughly.
5. Centrifuge the cell suspension at 280 × g for 3 min and aspirate the supernatant.
6. Resuspend Tet-MyoD-hiPSCs with primate ES cell medium supplemented with 10 μM of ROCK inhibitor.
7. Plate 1×10^5–1×10^6 cells to prepared matrigel- or collagen-I-coated 6-well plates. For each new hiPSC line and PB200-MyoD transgenic population or clone, carry out a pilot experiment to determine the optimal cell plating density for skeletal muscle cell differentiation (*see* **Note 3**).

8. Medium for differentiation is adjusted as follows (Fig. 4):
   - Differentiation day 1
     Primate ES cell medium supplemented with 1 μg/mL of dox.
   - Differentiation day 2–6
     5 % KSR/αMEM supplemented with 1 μg/mL of dox.
   - Differentiation day 7–14
     2 % horse serum/DMEM supplemented with 1 μg/mL of dox and 10 ng/mL of IGF-1.
9. Observe the cells. As shown in Fig. 5a, cells begin to change morphology after differentiation is initiated with dox. Rounded cell structure gradually changes to spindle-like structure from day 2 to day 7 of differentiation. Cell fusion to form multinucleated myocytes can be noted from day 9 of induction.
10. Evaluate the cells (*see* **Note 4**). Differentiated myocytes can be evaluated by immunohistochemistry as shown in Fig. 5b. It is important to confirm with skeletal muscle actin (SMA) antibody that obtained myocytes are actually skeletal myocytes. Yield can be calculated by counting the number of myosin heavy chain (MHC) positive cells per number of DAPI. Refer to Table 1 for primary and secondary antibody details. Further functional evaluation can be done by Western blotting or electric stimulation analysis (*see* **Note 5**).
11. Application. Disease modeling using Tet-MyoD-hiPSC-derived skeletal muscle cells will require custom assays appropriate to the disease in question. Moreover, in vitro recapitulation of disease pathology may require the application of suitable stresses to initiate disease pathogenesis. For example, in modeling Miyoshi myopathy (MM), we induced membrane injury by laser-irradiation of myocytes differentiated from

Table 1
Antibody list

First antibody	Source	Clonality	Dilution	Company
MHC	Mouse	Monoclonal	1/200	R & D
Skeletal muscle actin	Mouse IgM	Monoclonal	1/200	Acris
CKM	Rabbit	Polyclonal	1/100	Bioworld Technology
Second antibody			**Dilution**	**Company**
Alexa Fluor 488 conjugated goat-anti-mouse IgG			1/500	Invitrogen
Alexa Fluor 488 conjugated goat-anti-rabbit IgG			1/500	Invitrogen
Alexa Fluor 488 conjugated goat-anti-mouse IgM			1/500	Invitrogen

MM-patient derived hiPSCs [13]. We analyzed the latency time of membrane resealing by FM1-43 dye influx in order to assess the function of membrane repair. Alternatively, methods for inducing stress include electric stimulation, stretching, and chemical treatment.

4 Notes

1. If low cell viability is noted following transfection:
   (a) Refine the purity of plasmid DNA (endotoxin-free).
   (b) Carry out a preliminary experiment to determine the optimal ratio of transfection reagent to plasmid DNA.
   (c) Reduce the time for incubation of cells with transfection complexes from 24 h to 4, 8, or 12 h.
   (d) Transfection efficiencies can be confirmed by GFP expression within 12 h of dox addition. Perform the assay in a replica plate, as cells exposed to dox will express MyoD and may begin to differentiate.
2. How to maintain a pluripotent culture of Tet-MyoD-hiPSCs.
   Passage cells or subclone to obtain homogenous cell population. Adjusting concentration of Neo may be helpful.
3. Skeletal muscle cell differentiation.
   (a) If a low efficiency of differentiation is observed.
   Addition of Neo while maintaining Tet-MyoD-hiPSCs can help to prevent silencing of randomly integrated PB vectors.
   Determine the appropriate cell plating density for differentiation, as high cell confluency can negatively affect differentiation efficiencies.
   Subcloning is required to select the appropriate clone that has stable differentiation ability.
   Optimize the dox concentration appropriate for MyoD induction.
   (b) Undifferentiated cells remain throughout the differentiation process.
   Each cell line has different cell plating density for skeletal muscle cell differentiation. It is strongly recommended to carry out a preliminary experiment to determine the appropriate cell plating numbers for each cell line.
   Ensure that a pure population of Neo-resistant cells is used.
   Optimize the dox concentration appropriate for MyoD induction.

(c) Increased number of dead cells after dox addition. Confirm plating densities and optimize the dox concentration for appropriate MyoD induction.

(d) Differentiated cells can be cultured for up to 2 months. However, differentiated cells cannot undergo passage nor freeze/thaw processes.

4. How to evaluate yield and efficiency.

(a) Transduction efficiency of *MYOD1* in Tet-MyoD-hiPSCs can be evaluated based on GFP expression 1 day after the dox induction. With *piggyBac* vector, transduction efficiency is expected to be higher than 90 %.

(b) Immunohistochemistry will be the most applicable assay to evaluate yield and efficiency of differentiated myocytes. The number of MHC positive cells per DAPI stained nucleus indicates efficiency, mostly ranging 70–90 %. Since cultured cells are in a monolayer, yield can be calculated by directly counting myocytes on the plate.

5. Evaluating functionality of differentiated myocytes.

(a) Differentiated myocytes can twitch in response to electric stimulation [13].

(b) It is possible to detect the calcium influx according to the stimulation using Fluo-8, a calcium indicator.

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Using Human Induced Pluripotent Stem Cells to Model Skeletal Diseases

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Abstract

Musculoskeletal disorders affecting the bones and joints are major health problems among children and adults. Major challenges such as the genetic origins or poor diagnostics of severe skeletal disease hinder our understanding of human skeletal diseases. The recent advent of human induced pluripotent stem cells (human iPS cells) provides an unparalleled opportunity to create human-specific models of human skeletal diseases. iPS cells have the ability to self-renew, allowing us to obtain large amounts of starting material, and have the potential to differentiate into any cell types in the body. In addition, they can carry one or more mutations responsible for the disease of interest or be genetically corrected to create isogenic controls. Our work has focused on modeling rare musculoskeletal disorders including fibrodysplasia ossificans progressive (FOP), a congenital disease of increased heterotopic ossification. In this review, we will discuss our experiences and protocols differentiating human iPS cells toward the osteogenic lineage and their application to model skeletal diseases. A number of critical challenges and exciting new approaches are also discussed, which will allow the skeletal biology field to harness the potential of human iPS cells as a critical model system for understanding diseases of abnormal skeletal formation and bone regeneration.

Keywords: Human iPS cells, Mineralization, Fibrodysplasia ossificans progressiva, Ossification, Skeletal diseases, Directed differentiation

1 Introduction

Musculoskeletal conditions such as osteoporosis, fractures, and skeletal malformations are among the most frequently reported medical conditions in the USA and are the second-greatest cause of disability worldwide (1). Inherited skeletal disorders are among the most common genetic diseases (2) and affect 2.4 in 10,000 births with 23 % of the affected presenting as stillbirths and 32 % mortality in the first week of life (3). Adult osteoporosis alone affects over ten million people in the United States and results in over two million fractures each year (4). Being able to model these conditions in a human model system is one critical tool for developing therapies for these medically important diseases.

1.1 Major Challenges Hinder Our Understanding of Human Skeletal Diseases

Achieving a better understanding of human skeletal development has several major challenges:

First, the genetic factors underlying skeletal diseases are complex. Many of the traits and diseases we associate with the skeleton (e.g., height; osteoporosis) are multigenic in origin (5–7). In addition, some genes have distinct functions in humans that vary significantly from what occurs in model organisms such as rodents (8–10). Although model organisms provide valuable insights into biology, these genetic complexities indicate that having a continuous source of human tissues would be extremely valuable for understanding disease pathophysiology and translating our knowledge into new treatment strategies. Until recently, this has been a major hurdle since obtaining large quantities of primary tissues from humans can be very difficult or impossible.

Second, a surprisingly large number of severe skeletal and nonskeletal medical conditions remain "undiagnosed" with only rudimentary molecular understanding of the disease pathogenesis. Patients with these rare or orphan conditions often face diagnostic and treatment delays, which can be improved when the disease process is discovered. Importantly, research into some of these rare presentations has identified key pathways leading to breakthrough discoveries and medications that benefit the wider population (e.g., the role of *SOST* in regulating bone mass (11, 12)). This demonstrates that rare disease models can highlight important pathways and help address the unmet medical needs of more complex polygenic diseases such as osteoporosis.

Third, during the past several decades, bone researchers have focused on autologous cells such as mesenchymal stem cells (MSCs) or adult stem cells (e.g., adipose-derived stem cells) (13–16). These multipotent cell types are finding applications in regenerative therapies. However, isolating large numbers of primary cells remains difficult: one report showed that 30 ml of human bone marrow yielded only 7–22 $\times$ 10^6 phenotypic MSCs after 4 weeks of culture, with some samples requiring extended culture (17). In addition, multiple donors are needed as sources for different cell types (i.e., MSCs, endothelial cells, muscle stem cells), introducing different genetic backgrounds as a new confounder. This also decreases the likelihood that a composite allograft could be created from a single donor and increases the risk of allograft rejection if a multidonor allograft was used. Finally, other cell types abundant in bone, such as neurons or hematopoietic cells, cannot be easily generated from MSCs and thus their contributions are difficult to explore. Human iPS cells help address this challenge by allowing us to potentially generate any cell type of interest.

1.2 Pluripotent Cells Are Useful for Skeletal Research

Stem cells are defined as having two basic properties: the ability to self-renew and the potential to differentiate into one or more specialized cell types. Stem cells are critical for maintaining tissues that normally have high turnover such as skin and blood. However,

it is increasingly recognized that many organs, even ones with low proliferative capacity as can be found in the skeleton, contain tissue-specific stem cells that contribute to their growth and maintenance (18). These tissue-specific cells are typically multipotent and have limited differentiation potential to create only a subset of cell types. In contrast, cells in the mammalian early embryo are pluripotent and can contribute to any tissue in the body (19, 20).

Pluripotent cells such as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells are well suited for modeling human physiology, pathophysiology, and development since they can create any cell types that are needed, if the appropriate differentiation protocols are available. Although multipotent stem cells like MSCs or adult stem cells are valuable for studying skeletal diseases, pluripotent cells would allow us to generate lineages that may be critical for bone formation, but outside of the normal repertoire for lineage-restricted multipotent cells (i.e., neural crest cells, neurons, immune cells). Since many of the pathways that regulate skeletal development also have critical roles in other tissue types, human pluripotent cells can be used to study these functions in nonskeletal tissues. Furthermore, starting from a pluripotent cell potentially allows us to create a continuous supply of isogenic cell types, thus minimizing the effects of variations in genetic background that may occur with primary cells.

Human ES cells are derived from human embryos created from eggs fertilized in vitro (21, 22). Briefly, these cell lines are derived from blastocysts that have been plated on a tissue culture surface to allow the inner cell mass to expand. The surviving cells grow to create a renewable cell population. Cells that maintain a normal genetic background, and remain in a pluripotent state (i.e., do not differentiate into a terminal cell type), become an embryonic stem cell line. A number of human ES cell lines are currently available. NIH supports research using a select number of lines that have met specific quality control and ethical standards (http://escr.nih.gov).

1.3 Induced Pluripotent Stem Cells Are Useful for Modeling Human Diseases

The discovery of mouse (23) and human iPS cells by Shinya Yamanaka's laboratory in 2007 (24) revolutionized the stem cell field by providing a relatively straightforward method to create pluripotent cells from a differentiated cell source. iPS cells allow us to create unlimited numbers of isogenic cell types, providing a single, renewable source of human cells with a known genetic background. These purified cell populations allow new detailed genetic, biochemical, and functional studies not previously possible while providing a high level of long-term consistency for robust experiments and allowing us to link in vitro results to a patient phenotype. The recent finding of putative pluripotent cells in somatic tissues and the creation of stem cell banks from "superdonors" that are immune-compatible with multiple recipients (25) increase the possibility that our iPS cell studies will find rapid applications in tissue engineering as immune tolerance improves.

1.4 The Method of Reprogramming May Affect the Formation of iPS Cells

All of the current methods for creating iPS cells activate a pluripotency transcriptional network to convert a more differentiated cell into a pluripotent-like cell. Many iPS cell induction methods are now widely used and demonstrate that there are many roads to pluripotency. Methods include retroviral transduction (24); DNA constructs (26); nonintegrating episomes (27); nonintegrating Sendai viruses (28); nonintegrating modified mRNA transduction (29, 30), transposons (31), and small molecules (32). The field of reprogramming continues to innovate and many new methods are constantly being made available. Many of these techniques have been used to reprogram multiple types of terminally differentiated cells.

In our own studies using iPS cells created from patients with fibrodysplasia ossificans progressiva (FOP), we were concerned that activated BMP signaling by the FOP R206H ACVR1 mutation could adversely affect our ability to create FOP iPS cells since BMPs can induce human ES cell differentiation (33). In addition, prior reports using Sendai virus indicated that Sendai-derived FOP iPS cells were not able to maintain their pluripotent state (34). In our hands, we found that retroviral and episomal methods could create FOP iPS cells (35); however, the FOP iPS cells tended to lose their iPS cell-like morphology more often when cultured for long durations in feeder-free conditions. These results indicated that there are method-specific effects on iPS cell generation that are yet to be elucidated. It also indicates that if one method of reprogramming doesn't work, a different method may be more successful to compensate for factors (genetic or otherwise) that may influence iPS cell generation.

1.5 Directed Differentiation of Pluripotent Stem Cells

A tremendous library of protocols, too large to list here, is now available describing many ways to create differentiated cell lines from pluripotent stem cells. Over the past several years, new methods have been developed specifically for human iPS cells. These methods use different approaches, including robust small molecule-directed differentiation protocols (i.e., for cardiomyocytes (36), neurons (37), and endothelial cells (38)); expression of master transcription factors (i.e., for skeletal muscle (39)); and culture in less well-defined conditions that are known to favor the formation of specific lineages (i.e., for chondrocytes and mineralizing cells).

Directed differentiation methods continue to improve, particularly with the use of newer scaffolds and culture matrices. However, several factors need to be kept in mind: the specific protocols used in directed differentiation methods may be cell type specific; many commercial differentiation mediums are proprietary (i.e., osteogenic medium often contains BMPs, which may confound an experiment if the disease already affects the BMP pathway); and a detailed optimization process may be necessary when

applying the method to different cell lines. In addition, the use of specific medium conditions can make cocultures particularly challenging since the individual cell types may not survive together if the culture conditions are not compatible. Finally, human iPS cells appear to differentiate easily into immature cell types in a dish (40); however, more mature cell types may require advanced 3D or in vivo environments (41). Despite these limitations, the ability to make specific cell types from iPS cells carrying a specific disease mutation is exciting for disease modeling since in many cases, the specific cell types that are affected by the mutation are not easily identified or obtained from primary samples.

1.6 Osteogenic Differentiation and Mineralization

One of the major challenges when differentiating human iPS cells is to obtain a large "pure" population of skeletal-lineage cells that are functional in vitro and in vivo. Traditionally, cells are assayed for their potential osteogenic capacity by detecting mineralization, a relatively late step in the bone formation process. These types of protocols were mostly adapted from methods developed for MSCs, often used monolayers of cells cultured for 12–28 days, and were dependent on fetal bovine serum (35, 42). Most of these mineralizing or osteogenic medium contain β-glycerol phosphate, ascorbic acid, and dexamethasone (35, 43). There are also a number of commercial medium available for osteoblast differentiation or mineralization, mainly tested on MSCs. However, in each of these cases, it remains important to distinguish whether the end mineral deposition was associated with other indicators of osteogenesis such as collagen fibril deposition and increased expression of osteogenic genes (44).

Several protocols have recently emerged to differentiate human iPS cells and human ES cells into osteoblasts (Fig. 1). Most protocols utilize BMPs supplemented in the culture medium and directly added to the human iPS cells (45) since BMPs are powerful promoters of osteogenesis and regulate differentiation of pluripotent cells (33, 46). Although BMPs in the medium may be useful for directed differentiation, they may confound disease modeling depending on whether BMPs are part of the disease pathogenesis or phenotype. For example, our experience with the FOP iPS cells requires using mineralization medium without BMPs to study how the increased signaling activity of the mutated BMP receptor ACVR1 affects the function of our FOP iPS cell lines (35). Similar concerns may be important for conditions using small molecules or substrates. These compounds may interact with the genetic mutations found in the cell lines.

In vitro osteogenic differentiation of human iPS cells can also be performed using embryoid body (EB) methods. A number of these protocols were initially established in murine ES or iPS cells (47, 48). Several authors have reported that treating human iPS cell-derived EBs with all-trans retinoic acid, and subsequently

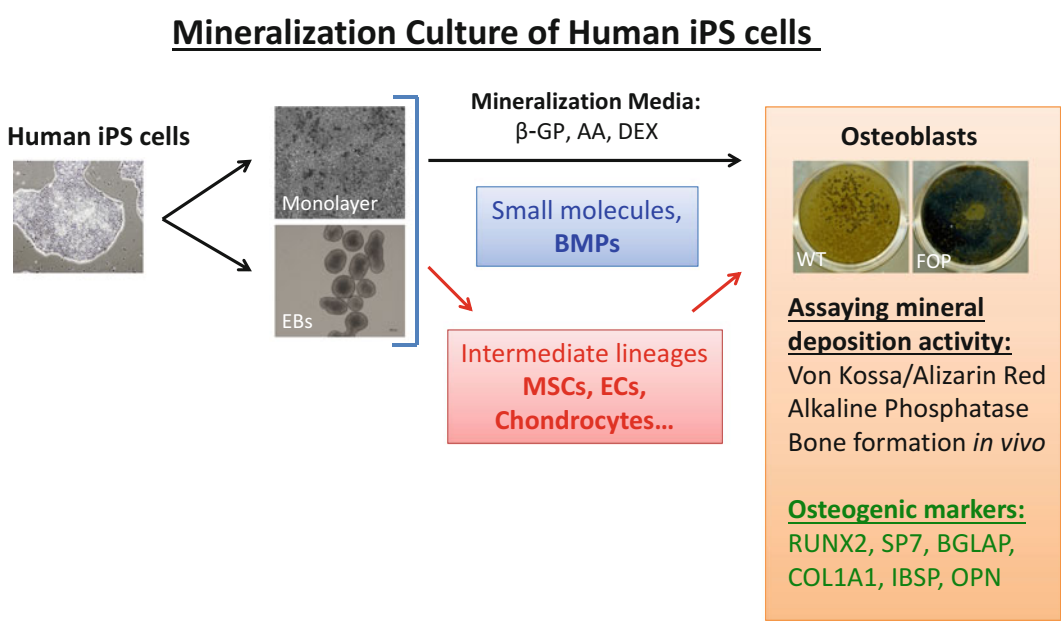

Fig. 1 Human iPS cells can be differentiated into osteoblasts via different protocols

culturing single-cell dissociated EBs in conditions that favor osteogenesis, can also lead to osteoblast differentiation in vitro and in in vivo implants (43).

A recent protocol described the use of small molecules under serum-free and feeder-free conditions to differentiate iPS cells into osteoblasts in a stepwise manner without the formation of EBs (49). These small molecules include GSK3 inhibitors (CHIR999021) to activate canonical WNT signaling cues to specify the differentiation toward mesodermal layers (50); a smoothened agonist (SAG), a hedgehog (HH) signaling activator which promotes early osteoblast differentiation in perichondrial cells consisting of osteochondroprogenitors (51); and TH (4-(4-methoxyphenyl)pyrido (4′,3′:4,5)thieno(2,3-b)pyridine-2carboxamide) for inducing osteoblast maturation (52). These defined conditions are extremely valuable as they will eventually provide more consistency, improve our ability to delineate osteoblast development and function, and facilitate more robust applications in drug screening and skeletal regeneration.

1.7 Assaying the Osteogenic Properties of Cell Lines

Determining if osteogenesis is increased in human iPS cell cultures can be challenging. As for mesenchymal stem cells, there are several surrogate markers that are commonly used including histology assays (staining for alcian blue to indicate cartilage; alkaline phosphatase to detect early mineralization activity; and calcium/phosphorous staining with von Kossa or alizarin red to detect mineral deposition). The direct assessment of osteoblast number also

remains challenging mostly because of the dearth of cell surface markers that clearly identify the specific lineages and stages of skeletogenesis. Recently, several groups have created genetically marked reporters for use in human ES and iPS cells (53, 54). These types of constructs will be extremely valuable for assaying osteoblast and chondrocyte formation directly as well as identifying additional markers for these skeletal cell lineages.

One emerging direction is the expanded use of 3D cell culture methods for studying the osteogenic micro-niche as well as combining human iPS cells with in vivo bone regeneration models in rodents. For example, critical-sized cranial defects that do not spontaneously heal can be implanted with human iPS cells seeded onto scaffolds constructed from hydroxyapatite-coated poly-L-lactic acid engineered to release BMP2. The implanted cells can facilitate the repair of these bone lesions, suggesting that human iPS cells directly contribute to osteogenesis in vivo when implanted in the right setting (45). This type of model can be very useful to acquire terminal cell fate by pluripotent cells in an in vivo setting as a complement to in vitro functional analysis.

1.8 Directed Differentiation into Other Cell Lineages Found in Bone

One significant benefit for using human iPS cells to study the skeleton is the potential ability to create all of the cell types present in bone, including endothelial cells (38), osteoclasts (55), macrophages (56), and skeletal muscle cells (39). A promising strategy is to use pluripotent cells as a source of intermediate cell types or osteoprogenitors, such as MSCs (57). Several papers have described generation of MSCs from human iPS cells using a variety of different methods, including EB formation (58, 59), small molecules such as TGFβ pathway inhibitors (60), synthetic polymer substrates (61), or coculture with murine cells (62, 63). These methods are promising and will benefit from new methods that mark tissue-specific MSCs and a better understanding of the different subsets of tissue-specific MSC-like cells. In addition, the finding that endothelial cells expressing the FOP ACVR1 R206H mutation can undergo endothelial-to-mesenchymal transition (EndoMT) to form MSC-like cells and mineralize (64) suggests that there are likely multiple routes to create osteogenic precursors.

1.9 Future Directions

Human iPS cells are a promising way to generate human cell types from patients with genetic conditions, for disease modeling, for drug screening, and for tissue engineering. iPS cells provide an important complement to adult stem cells and mesenchymal stem cells by allowing the creation of a broader array of cell types.

More widespread application of iPS cells to musculoskeletal diseases is on the forefront and will be facilitated by the development of better directed differentiation protocols that exhibit high yield, cellular uniformity, and ease of use, particularly for lineages directly relevant to musculoskeletal tissues. New approaches using

directed nucleases such as TALENs (65) and CRISPRs (66) also show great promise as a way to induce point mutations and reporter constructs into human genomic DNA. These methods will help speed the process of genomic targeting for both pluripotent and differentiated cells on a uniform background or to create corrected isogenic cell lines as research controls. Finally, new marker and reporter lines for identifying skeletal gene expression, cell surface markers for purifying mesenchymal lineages, and libraries of diseased and genetically corrected human iPS cells will be extremely valuable tools for advancing the application of pluripotent stem cells for musculoskeletal diseases.

2 Materials

2.1 Feeder Cell Preparation

1. SNL Feeder cells: Mouse fibroblast STO cell line (67), which carries the neomycin resistance and murine leukemia inhibitory factor (LIF) genes—S, STO; N, neomycin resistance; L, LIF gene.
2. Gelatin 0.1 % (Sigma #G1393). Store at 4 °C.
3. Feeder Cell Maintenance Medium:

DMEM with Glutamax	LifeTechnologies, #10566
10 % FBS	Hyclone, characterized grade, #SH30910.03 Lot#AYK176955
Pen/Strep 10 U/ml	LifeTechnologies, #15140
Sodium Pyruvate 1 mM	LifeTechnologies, #11360

4. Feeder Cell Freezing Medium:

DMEM with Glutamax	LifeTechnologies, #10566
10 % FBS	Hyclone, characterized grade, #SH30910.03 Lot#AYK176955
Pen/Strep 10 U/ml	LifeTechnologies, #15140
Sodium Pyruvate 1 mM	LifeTechnologies, #11360
10 % DMSO	Sigma, #D2650

2.2 Human iPS Cells Maintenance on Feeders and Transfer onto Matrigel

1. mTesr Medium (StemCell Technologies cat #05850). Supplement is stored at −20 °C. Thaw Supplement at 4 °C overnight. Store reconstituted medium at 4 °C for up to 2 weeks maximum.
2. BD Matrigel Basement Membrane Matrix—GFR, High concentration (BD 354263). Keep Matrigel frozen at −80 °C in aliquots of 500 μl in 50 mL conical tubes. Thaw vial overnight

at 4 °C and dilute with KO DMEM (LifeTechnologies, #19829) to a concentration of 300 μg/ml. Note: Matrigel should always be kept cold as it will polymerize at room temperature and then will not dissolve in KO DMEM.

3. Y-27632 ROCK Inhibitor (EMD cat #688000). Resuspend in DMSO and store in 10 mM aliquots at −20 °C.
4. Human iPS Cell Freezing medium.

90 % mTeSR, reconstituted with supplement	
10 % DMSO	Sigma, #D2650
10 μM Y-27632 ROCK Inhibitor (EMD cat #688000) in DMSO	

2.3 Osteoblast Differentiation from Human iPS Cells

1. mTeSR reconstituted with supplement (Stem cell Technology #05850), stable for 2 weeks at 4 °C, warm up only aliquots to room temperature prior to use.
2. Y-27632 (ROCK Inhibitor, (EMD cat #688000)).
3. Accutase (Millipore #SCR005).
4. Gelatin 0.1 % (Sigma # G1393).
5. Ascorbic Acid 2 Phosphate (Sigma # A8960), 50 mg/ml in sterile water, stable for 1 week at 4 °C.
6. Dexamethasone water soluble (Sigma #D2915), 4 mM stock solution in sterile water. Store stock solution at −20 °C, stable for 6 month. Store 4 μM working solution at 4 °C.
7. Glycerol-2-Phosphate (Sigma #G9422), 1 M stock solution in sterile water. Store at 4 °C.
8. β-mercaptoethanol (Sigma #M6250) 0.143 M in sterile water, stable for 2 weeks at 4 °C.
9. Osteoblastic base medium (OB):

KO DMEM	LifeTechnologies, #19829
20 % FBS Characterized	Hyclone, characterized grade, #SH30396.03 Lot#AVC66310, Filter sterilized
2 mM Glutamax	LifeTechnologies, #35050
1 % NEAA	Nonessential Amino-Acids, LifeTechnologies, #11140
0.1 mM β-mercaptoethanol	Sigma #6250
10 mM Dexamethasone	Sigma #D2915
10 mM glycerol-2-phosphate	Sigma #G9422

2.4 Von Kossa/ Alcian Blue Staining

1. Silver Nitrate solution (Fisher #S181-25), 0.05 g/ml in distilled water. Must be prepared and stored in the dark at room temperature. Filter the solution with funnel and filter paper before use.
2. Sodium Carbonate-Formaldehyde solution, 0.05 g/ml sodium carbonate (anhydrous, Fisher#S263-500), 0.37 % formaldehyde solution (Fisher #F79-500), prepared in distilled water. Store at room temperature.
3. 1 % Alcian Blue solution (Sigma, #A3157) pH 2.5 in 3 % acetic acid. Filter through a 0.4 μm syringe filter just prior to use. This solution is stable for up to 6 months. The final dye concentration is approximately 0.65 %. Store at room temperature.

2.5 Alkaline Phosphatase Staining

1. 95 % EtOH.
2. BCIP/NBT substrate solution (Sigma, #B5655). Keep the substrate solution protected from light. Can be at RT for 1 h max just prior to use.

3 Methods

3.1 Feeder Cell Preparation

1. Thaw one vial of 1.5×10^6 of nonirradiated STO ECACC SNL6/7 cells and plate onto one T225 with 50 ml Feeder Medium. Culture 4–5 days, no medium change needed.
2. Once the cells are 90 % confluent (small cells, just touching together), split 1:10 into ten new T225s (or use the multilayer flasks, such as BD353144). Culture 4–5 days, until confluent.
3. Harvest cells by dissociating with 4 ml trypsin/T225 for 2–5 min. Quench with 4 ml Feeder Medium. Pool five flasks together (40 ml total). Rinse the five flasks with 12 ml feeder medium, recovering >10 ml. Repeat for other set of five flasks
4. Count cells. Expected yield is $240–300 \times 10^6$ cells total for ten T225 flasks. Freeze down vials of nonirradiated cells here, if needed. Irradiate remaining cells for 60 Gy total dose. Note that many commercial sources use/suggest 40 Gy. We have had a few cases of breakthrough growth at low doses. However, do not overdose, as at >80 Gy the cells have low viability and will not support iPS cell cultures.
5. Spin down cells at $200 \times g$ for 10 min. Aspirate medium and resuspend cells in Freezing Medium. Freeze in 1 ml aliquots of 3×10^6 cells slowly (1 °C/h, using cell freezing cooler) and store long term in liquid nitrogen tank.
6. If needed, there are enough leftover cells in the TC flasks that new medium can be added on and SNLs re-expanded once (i.e., don't throw the flasks away if you want to do a second

expansion. More than two expansions reusing the same flasks are not recommended).

3.2 Human iPS Cells Maintenance on Feeders and Transfer onto Matrigel

1. *SNL Feeder Cell Thawing*
   (a) Add 1 mL of 0.1 % gelatin to each well of a six-well plate and incubate the six-well plate with gelatin at 37 °C for 15 min.
   (b) Thaw a vial of irradiated SNLs (3×10^6 cells per vial) and resuspend in MEF medium.
   (c) Remove the gelatin from the six-well plate. Add 2 mL of the cell suspension to each well of the plate and incubate at 37 °C.
   (d) Wait at least 24 h before seeding iPS cells (**Note 1**).
2. *Thawing human iPS cells.*
   (a) Prepare an aliquot of complete mTeSR + 10 μM ROCK inhibitor.
   (b) Thaw human iPS cells in a 37 °C water bath and resuspend KO DMEM.
   (c) Centrifuge for 3 min at 100 × *g*, aspirate the supernatant, and resuspend the iPS cells in 2 ml complete mTeSR +10 μM ROCK inhibitor (final concentration).
   (d) Remove the medium of the SNLs and plate cells into one to three wells of a six-well SNLs plate.
3. *Passaging iPS cells (generally 4–5 days after thawing or seeding).*
   (a) Prewarm complete mTeSR supplemented with ROCK inhibitor final concentration 10 μM.
   (b) Remove the medium from iPS cells and rinse with DPBS. Add 0.5 mL of accutase to each well and incubate at 37 °C for 3 min.
   (c) While the cells are incubating, take a six-well plate with SNLs from the 37 °C incubator and remove the medium.
   (d) Transfer the iPS cells to a 15 mL conical tube and centrifuge for 3 min at 100 × *g*.
   (e) Remove supernatant and resuspend the iPS cells in 2 mL complete mTeSR supplemented with ROCK inhibitor. Split the cells (typically 1:10, but high splits are generally not tolerated well) to a new plate of SNLs with mTesr and ROCKI; adjust the split ratio for each cell line.
   (f) Change medium every 24 h with 2 mL of complete mTeSR (no ROCKI).
   (g) Human iPS cells should be split once they are 80 % confluent, generally 4–5 days after thawing or splitting. It is important to check the phenotype and make sure that iPS cells are not differentiated. (Human iPS cells may be plated

on SNLs for maintenance or on Matrigel for further differentiation experiments, if desired. *See* below.)

4. *Matrigel-coated plates preparation.*
   (a) Prepare Matrigel plate. Frozen aliquots of Matrigel need to be thawed overnight at 4 °C.
   (b) Dilute Matrigel with *cold* KO DMEM (final concentration approx. 300 μg/ml). (Check the concentration based on the batch). Diluted Matrigel can be kept in the conical at 4 °C for 1–2 weeks.
   (c) Add one 1 ml of diluted Matrigel per well of six-well plate or 3 ml per 10 cm plate. Place in the incubator at 37 °C for 40 min.

       Note: if the plates are left in the incubator for longer, it is important to make sure that the wells don't dry out. The coated plates should be used the same day they were coated.

   (d) Before use, aspirate Matrigel, then immediately seed the iPS cells.
5. *Freezing Human iPS cell*
   (a) Centrifuge cells at 200 × *g* for 10 min. Aspirate medium and resuspend cells in Human iPS cell Freezing Medium. Freeze in 1 ml aliquots cooling cells slowly (1 °C/h, using cell freezing cooler) and store long term in liquid nitrogen tank.

3.3 Osteoblast Differentiation from Human iPS Cells

1. Culture human iPS cells in 10 cm plates to 85 % confluence. One 10 cm plate should allow preparing two 24-well plates.
2. Day 0: Gelatin coat plates based on expected number of cells. For 24-well plates, use 400,000 cells/well. Wait at least 30 min for gelatin to coat the surface at 37 °C.
3. iPS cells are washed with DPBS and then incubated with 2 ml of prewarmed Accutase for 3 min at 37 °C. To completely detach the cells you may tap the side of the plate or scrap them.
4. Add 5 volumes of KO DMEM (approx. 10 ml), gently pipette up and down to detach all the iPS cells, transfer in a 15 or 50 ml conical tube, and centrifuge at 100 × *g* for 3 min.
5. Resuspend the iPS cells in 15 ml 37 °C prewarmed mixed medium 90 % OB Medium (OB Base medium supplemented with 50 μg/ml of Ascorbic acid) / 10 % mTeSR, supplemented with 10 μM ROCK Inhibitor) for 1 × 10 cm^2 dish (increase if more dishes have been pooled).
6. Count cells. Remove the gelatin from the receiving plate.
7. Based on the amount of live cells, plate 400,000 cells/well in 1 ml of mixed medium and place the plates at 37 °C, 5 % CO_2.

8. Day1: Change medium to Osteoblastic base medium extemporaneously supplemented with 50 μg/ml of Ascorbic Acid.
9. Medium is changed every other day until day 24. The medium should be changed very carefully as the cells may start to peel off around day 10.

3.4 Von Kossa/ Alcian Blue Staining for Tissue Culture (Note 2)

1. All steps of this protocol should be performed in a chemical hood.
2. Remove medium from wells and gently rinse with DPBS.
3. Fix the cells with 4 % PFA for 15 min and rinse three times with water. Rinse wells carefully, as any residual PFA may cause the subsequent staining steps to be spuriously positive.
4. Stain in Silver Nitrate solution for 15 min in dark (wrap with aluminum foil) and wash three times with distilled water, 1 min each.
5. Develop in Sodium Carbonate-Formaldehyde solution for 2 min (time is critical) and wash two times with distilled water, 1 min each. At this time plates may be air-dried and photographed or continue with the Alcian Blue staining.
6. Optional alcian blue staining: Add 1 % Alcian Blue solution (pH 2.5) for 1 h (shorter is likely OK) and wash two times with water, 1 min each.
7. Air-dry plate and photograph or scan.

3.5 Alkaline Phosphatase Staining

1. Aspirate medium from the cell culture plates to fix and wash once with PBS 1×.
2. Fix the cell by adding 95 % EtOH and incubate at RT for 10 min minimum (1 h max).
3. Rinse three times with DPBS and add NBT/BCIP substrate solution to each well.
4. Incubate the cell culture plate for 5–10 min at 37 °C, 5 % CO_2.
5. Rinse three times with water and dry in open air.

4 Notes

1. *Feeder cell preparation.*
   Note: It is not required to change the medium every day since the cells are irradiated and will not grow. However, if they are going to stay on the plate for an extended period of time, it is recommended to change the medium every 3–4 days.
2. *Alcian blue staining.*

Generally, the two stains should be done separately at first to make sure the process is working and robust.

Alcian blue pH can be adjusted to stain different mucins:

pH 2.5 = most acid mucins (except strongly sulfated group) (blue).

pH 1.0 = only weekly and strongly sulfated acid mucins.

pH 0.2 = strongly sulfated acid mucins only.

Mineral = black

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Modeling Cardiovascular Diseases with Patient-Specific Human Pluripotent Stem Cell-Derived Cardiomyocytes

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Abstract

The generation of cardiomyocytes from human induced pluripotent stem cells (hiPSCs) provides a source of cells that accurately recapitulate the human cardiac pathophysiology. The application of these cells allows for modeling of cardiovascular diseases, providing a novel understanding of human disease mechanisms and assessment of therapies. Here, we describe a stepwise protocol developed in our laboratory for the generation of hiPSCs from patients with a specific disease phenotype, long-term hiPSC culture and cryopreservation, differentiation of hiPSCs to cardiomyocytes, and assessment of disease phenotypes. Our protocol combines a number of innovative tools that include a codon-optimized mini intronic plasmid (CoMiP), chemically defined culture conditions to achieve high efficiencies of reprogramming and differentiation, and calcium imaging for assessment of cardiomyocyte phenotypes. Thus, this protocol provides a complete guide to use a patient cohort on a testable cardiomyocyte platform for pharmacological drug assessment.

Keywords: Cardiomyocytes, Disease modeling, Human induced pluripotent stem cells, Calcium imaging

1 Introduction

The heart is uniquely at risk to genetic disease due to the dependence on structural proteins for function and requirement for high levels of mitochondrial activity. Techniques to model human cardiac diseases have progressed significantly in the last five years with the development of patient-specific human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) (1), which has been shown to accurately recapitulate a large number of genetic and environmentally acquired cardiac diseases (2, 3).

To reach this point, significant advances are required in human pluripotent culture, reprogramming, and cardiac differentiation. For the reprogramming of human somatic cells into hiPSCs, progress has been made in the methodology for gene delivery, starting with retroviral and lentiviral expression and progressing through self-replicating episomal plasmids to a single *c*odon *o*ptimized *m*ini *i*ntronic *p*lasmid (CoMIP) (4) that is demonstrated here.

In CoMiP, the human *OCT4*, *SOX2*, and *KLF4* cDNA sequences have been replaced with those most suited for high level expression, and a plasmid has been used with a minimal-size backbone to enhance transfection efficiency. This technique allows for reprogramming without the integration of exogenous DNA sequences, thus maintaining the integrity of the target cell genome.

A second major development has been the discovery that a simple, chemically defined, serum/albumin-free medium consisting of just eight components (E8) can be used to culture hiPSCs (5), which can be modified to be compatible for reprogramming (E7). The advent of this media substantially improves the quality of hiPSC cultures (i.e., by eliminating spontaneously differentiating cells) and reduces the cost and complexity of reprogramming and culture. Recently, it has been demonstrated that hiPSC-CM differentiation can be performed using a chemically defined medium and small molecules, without the need for serum or growth factors, as demonstrated here. This methodology has proven to be reliable and reproducible for the differentiation of a large number of hiPSC lines (6). Finally, it has been demonstrated that immunofluorescent staining for TNNT2 (troponin T) and ACTN2 (α-actinin) can detect a known structural phenotype, and that we can detect a functional phenotypes using calcium imaging with Fluo-4AM.

Here we demonstrate that by combining all three of these advances (i.e., CoMIP, chemically defined reprogramming, and chemically defined differentiation), somatic cells can be isolated from a patient, reprogrammed to hiPSCs, and differentiated to hiPSC-CMs. The cells can be phenotypically characterized using immunofluorescence and calcium imaging. The aim of this study is to reproduce the data published earlier using lentiviral-derived dilated cardiomyopathy (DCM) hiPSCs (7), now derived with a non-integrating technique (CoMiP). We concentrated on this disease model as it is one of the first hiPSC-CM disease models that demonstrated a phenotype that was not just electrophysiological. We show that the published DCM phenotype caused by a mutation of *TNNT2* (R173W) is independent of the integrated lentivirus, and that this mutation causes the phenotypic perturbations previously seen (i.e., elimination of sarcomeric alignment and reduction in calcium handling).

2 Materials

2.1 Patient Fibroblast Isolation and Growth

1. Lidocaine HCl 1 % and epinephrine 1:100,000 injection.
2. 1 mL SafetyGlide TB syringe.
3. 3 mm Tri-punch disposable skin punch biopsy punch.
4. Sterile non-latex gloves.

5. Alcohol wipes.
6. ChloraPrep One-Step (2 % chlorhexidine gluconate and 70 % isopropyl alcohol).
7. Chlorhexidine gluconate cloth.
8. 15 and 50 mL polypropylene conical tubes.
9. MycoAlert kit.
10. Fibroblast medium (*see* formula 500 mL DMEM high glucose, with GlutaMAX, HEPES, 10 % FBS, filter-sterilized).
11. Collagenase II solution (*see* formula 50 mg collagenase II, 50 mL DMEM, filter-sterilized).
12. Matrigel-coated 6-well plates, 100 mm dishes and T225 flasks (*see* protocol).
13. Dimethyl Sulfoxide (DMSO).
14. Fetal Bovine Serum (FBS).
15. Cryovials.
16. CoolCell LX.

2.2 Somatic Cell Reprogramming

1. CoMiP plasmid (1 μg/μL, provided by S. Diecke and J. Wu upon request).
2. TrypLE Express.
3. Fibroblast medium (*see* formula 500 mL DMEM high glucose, with GlutaMAX, HEPES, 10 % FBS, filter-sterilized).
4. E7 medium (*see* formula Essential 6, 100 ng/mL FGF2).
5. Essential 8 medium.
6. E8Y medium (*see* formula Essential 8, 10 μM Y27632).
7. Neon transduction device with 100 μL tips.
8. Sodium butyrate.
9. L-ascorbic acid 2-phosphate.
10. Matrigel-coated 100 mm dishes and 6-well plates (*see* protocol).
11. 0.5 mM EDTA.
12. Bambanker.
13. Cardiomyocyte Differentiation Kit.
14. RPMI 1640 (without glucose).
15. B-27 with insulin supplement.

2.3 Cell Dissociation and Calcium Imaging

1. TrypLE Express.
2. 100 μm cell strainer.
3. 8-chamber coverslips.
4. Fluo-4 AM.

5. Pluronic F-127, 0.2 μm filtered (10 % solution in water).
6. Tyrode's salts.

2.4 Equipment

1. Cell culture incubator capable of 37 °C, 5 % CO_2, 85 % relative humidity.
2. Automated cell counter.
3. Centrifuge −86 °C ultralow temperature freezer.
4. Liquid nitrogen storage.
5. Inverted cell culture microscope (such as LEICA DMi1).
6. Confocal microscope (such as Zeiss LSM 510Meta).

3 Methods

3.1 Patient Fibroblast Isolation, Growth, and Cryopreservation

1. Obtain local Institutional Review Board (IRB) approval that complies with local and national laws and guidelines.
2. Informed consent forms signed by patient.
3. To be performed by MD or RN: Swab area to be biopsied (hip/upper cheek area, not exposed to sun) twice with iodine, inject lidocaine, using sterile gloves, take two 3 mm skin punch biopsies and place in ~5 mL of fibroblast medium.
4. Transport back to lab in a 15 mL Falcon tube.
5. Fill two wells of a 6-well plate with 2 mL of 200 U/mL collagenase II and transfer each skin biopsy from Falcon tube to a well using a P1000 tip. Using a round blade scalpel, cut the biopsy into approximately 0.5–1 mm pieces.
6. Incubate for 4 h at 37 °C, during this time prepare Matrigel-coated plate (*see* protocol).
7. Transfer cells/collagenase to a 15 mL Falcon tube, top up with fibroblast medium, centrifuge at 200 × *g* for 4 min, resuspend pellet in 2 mL fibroblast medium, and transfer each biopsy to one well of a Matrigel-coated 6-well plate.
8. Every 3–4 days remove the medium and transfer to a 15 mL Falcon tube, top up the well with 2 mL fibroblast medium, centrifuge the spent medium, and return any pelleted cells to the well.
9. Cell clumps will attach after ~5 days and after ~15 days wells will be ~50 % confluent and ready to passage.
10. To passage, aspirate medium, add 1 mL per well TrypLE Express and incubate at 37 °C for 2 min. Transfer dissociated cells to a 15 mL Falcon tube and top up with fibroblast media, and centrifuge at 200 × *g* for 4 min. Resuspend pellet in 45 mL fibroblast media and transfer cells to a Matrigel-coated T225 flask. Change media every other day.

11. After ~5–7 days, cells will be confluent and ready to be cryopreserved.
12. Passage cells as above, centrifuge and resuspend cells in 10 mL cold (4 °C) 10 % DMSO/90 % FBS.
13. Aliquot cells at 1 mL per cryovial, 10 vials total, these will be passage 2 when thawed.
14. Transfer vials to CoolCell and place at −86 °C overnight.
15. Transfer vials to liquid nitrogen tank (*see* **Note 1**).

3.2 Coating Tissue Culture Plates and Flasks with Matrigel

1. Thaw a bottle of growth factor-reduced Matrigel overnight at 4 °C and store it at 4 °C. There is no need to aliquot or leave it on ice.
2. Add 250 μL of Matrigel to 50 mL of 4 °C DMEM/F12 in a 50 mL Falcon tube. Return the Matrigel bottle to 4 °C quickly as it will gel at >10 °C.
3. Mix the tube by inversion and plate at 1 mL per well of a 12-well plate, 2 mL per well of a 6-well plate, or 45 mL into a T225 flask.
4. Place plates/flasks at 37 °C for at least 30 min. Plates/flask may be kept here at 37 °C for up to 4 weeks.
5. Before use, aspirate diluted Matrigel. Washing with D-PBS is not required.

3.3 Reprogramming of Fibroblasts with CoMiP (Fig. 1)

1. The target fibroblasts cell line should be thawed and cultured for at least 3 days before starting the reprogramming experiment.
2. Thaw a vial of fibroblasts for ~1 min in a 37 °C water bath, transfer cells to a 15 mL Falcon tube and top up to 10 ml with fibroblast medium, centrifuge at 200 × *g* for 4 min and plate in a 100 mm Matrigel-coated dish. Grow to confluence (~2–3 days) and passage with TrypLE Express as above.
3. Count cells using an automated cell counter, place 1.1×10^6 cells in a 15 mL Falcon tube and centrifuge at 200 × *g* for 4 min.
4. Prepare the Neon transfection device following the manufacturer's instructions using Buffer E2.
5. Aspirate the supernatant and resuspend the cell pellet in 100 μL Neon Buffer R; add 10 μg of CoMiP plasmid and resuspend gently. Load the cells mixed with the DNA into the 100 μL Neon transfection tip (make sure to avoid introducing air bubbles in the tip as this will result in poor transfection efficiency and reduced viability of the cells).

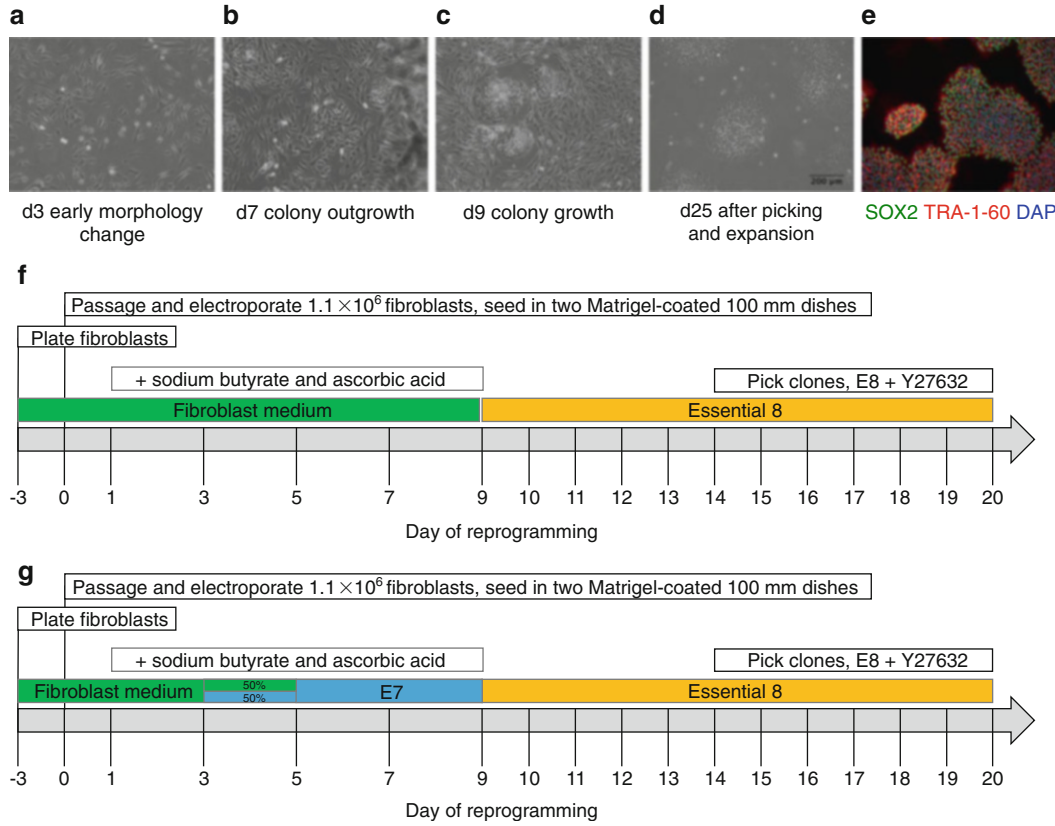

Fig. 1 Stepwise protocol for the reprogramming of fibroblasts to human induced pluripotent stem cells (hiPSCs) using a single codon-optimized mini-intronic plasmid (CoMiP). Day of reprogramming represents the days at which the medium is changed. (**a**–**d**) Phase-contrast images of the five major stages of reprogramming demonstrating the progression from the fibroblast morphology, through early hiPSC colonies and cells ready for picking, and cells at passage 1. (**e**) Immunofluorescent staining of CoMiP-derived hiPSCs for the common pluripotency markers SOX2 and TRA-1-60. (**f**–**g**) Two different strategies for reprogramming of fibroblasts: one approach uses continued culture in fibroblast medium (containing FBS) up until day 9 of reprogramming before switching to E8 medium, and a second approach uses a gradual transfer from fibroblast medium through chemically defined E7 medium (E8 without TGFβ1) to E8 medium

6. Perform the transfection using 100 μL Neon tip, making sure it is properly inserted into the transfection tube (*see* Instruction manual) under the following settings: 1,600 V, 10 ms, and 3 pulses (transfection efficiency should be more than 50 %).
7. Mix the transfected cells with 30 mL of fibroblast medium.
8. Plate the cells equally distributed onto two Matrigel-coated 100 mm plates (5.5×10^5 cells); name the plates Plate 1 and Plate 2.
9. Day 1: Change medium on both plates to fibroblast medium with sodium butyrate (NaB, 200 μM) and L-ascorbic acid 2-phosphate (AA2-P, 64 μg/mL)

10. Day 3: Change medium on Plate 1 to fibroblast medium with NaB and AA2-P. Change medium on Plate 2 to 50 % fibroblast medium; 50 % E7 with NaB
11. Day 5: Change medium on Plate 1 to fibroblast medium with NaB and AA2-P. Change medium on Plate 2 to E7 medium with NaB.
12. Day 7 onwards: Change medium on Plate 1 (fibroblast medium with NaB and AA2-P) and plate 2 (E7 medium with NaB) every other day
13. Around day 9 onwards: After you recognize the first colonies, switch to E8 medium and change medium every day.
14. Around day 14–20, the hiPSC colonies should be big enough for manual picking under the microscope. Pick six individual hiPSC clones and transfer them into six different wells of a Matrigel-coated 12-well plate in E8 medium with 10 μM Y27632.
15. If you have problems detaching the hiPSC colonies from the surrounding fibroblasts, you can scrape the fibroblasts away to make space for the outgrowing hiPSC colony. After two days, the free hiPSC clone should be large enough for easy picking.
16. Once cells are picked they can be clump passaged with 0.5 mM EDTA, gradually increasing the split ratio at each passage from 1:3 to 1:12 (*see* **Note 2**).

3.4 Long-Term Culture of hiPSCs

1. Ideally cells should have reached 75–95 % confluence in 4 days. Adjust split ratio ~1:6 to 1:12 to achieve this, as higher split ratios (1:12) will result in more efficient differentiations.
2. Aspirate culture medium from well.
3. Add ~950 μL of 0.5 mM EDTA per well, incubate for 6 min at RT (in hood)
4. During this time, set up a 50 mL Falcon tube with E8T (i.e., for a 1:12 split add 24 mL for 1 well).
5. Remove EDTA from the well with a P1000 tip, eject tip.
6. Using a P1000 tip, add 1 mL of the E8T medium from the Falcon tube containing E8T and blast against cell surface to dissociate cells. Cells should come off easily. Turn plate 180° to detach all cells. Transfer cell suspension to the Falcon tube containing E8Y.
7. Aspirate un-gelled Matrigel from Matrigel-coated 6-well plate.
8. Invert capped E8T tube containing cells to mix and plate out cells at 2 mL per well. Use no larger than a 10 mL pipette to improve consistency in number of cells per well.
9. After 24 h, aspirate used medium and replace with E8.
10. Repeat media change at 48 and 72 h.

11. At 96 h either passage cells or begin differentiation (*see***Note 3**).
12. Staining of hiPSCs for pluripotency markers can be performed as described in the Life Technologies Pluripotent Stem Cell 4-Marker Immunocytochemistry Kit.

3.5 Cryopreservation of hiPSCs

1. When cells are 75–95 % confluent (96 h), aspirate culture medium from well, add ~950 μL of 0.5 mM EDTA per well, and incubate for 6 min at RT (in hood).
2. Using a P1000 tip, add 1 mL of cold (4 °C) Bambanker and blast against cell surface to dissociate cells. Cells should come off easily.
3. Turn plate 180° to detach all cells. Aliquot cells at 1 mL per cryovial.
4. Transfer vials to CoolCell and place at −86 °C overnight.
5. Transfer vials to liquid nitrogen tank.

3.6 Cardiac Differentiation

1. Grow hiPSCs to 80 % confluence in 96 h as described above (*see* **Note 4**).
2. Day 0: Aspirate medium in wells and replace with 2 mL per well of Cardiomyocyte Differentiation Medium A (Fig. 2).
3. Day 2 Aspirate medium in wells and replace with 2 mL per well of Cardiomyocyte Differentiation Medium B.
4. Day 4 then every other day up to day 15: aspirate medium in wells and replace with 2 mL per well of Cardiomyocyte Maintenance Medium.
5. Contracting cardiomyocytes should appear from day 9 to 11 of differentiation.
6. Cardiomyocytes can be purified using metabolic selection by replacing the Cardiomyocyte Maintenance Medium with RPMI 1640 no glucose supplemented with 2 % B-27 for day 10 to day 15.

3.7 Dissociation of Cardiomyocytes

1. Aspirate medium from wells.
2. Add 1 mL of TrypLE Express and incubate for 10–15 min at 37 °C.
3. Pipette up and down with a P1000 to dislodge cells and to break up aggregates. Avoid forming bubbles.
4. Transfer cells into a 15 mL Falcon tube and top up with Cardiomyocyte Maintenance Medium. Centrifuge at 200 × *g* for 5 min.
5. Resuspend in 1 mL Cardiomyocyte Maintenance Medium and pipette up and down 10 times to release single cells, top up to ~ $>1 \times 10^6$ cells/mL. One well of a 6-well plate will provide ~1–3 × 10^6 cells.

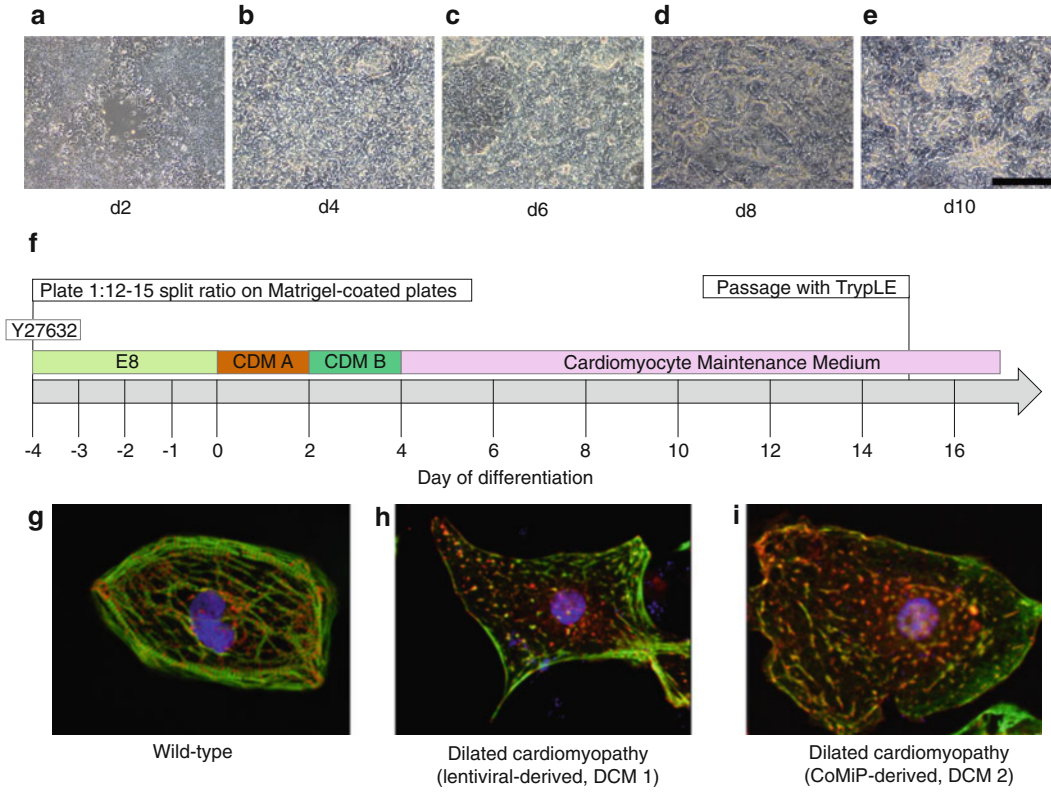

Fig. 2 Stepwise protocol for the differentiation of human pluripotent stem cells into cardiomyocytes (hiPSC-CMs). (**a**–**e**) Phase-contrast images of the five major stages of differentiation from the initial epithelial to mesenchymal transition through the acquisition of the refractory cardiomyocyte morphology. (**f**) Timeline of cardiac differentiation. Day of differentiation represents the days at which the medium is changed. (**g**–**i**) Immunofluorescent images of cardiomyocytes from three hiPSC lines stained for the cardiomyocytes structural markers TNNT2 (troponin T, *green*) and ACTN2 (α-actinin, *red*). (**g**) Cardiomyocytes from a wild-type hiPSC line demonstrating aligned sarcomeres. (**h**) Cardiomyocytes from a hiPSC line (DCM 1) derived from a DCM patient with a *MYH6* mutation, which we reprogrammed using lentivirus (as published in ref. (7)) showing punctate, non-aligned TNNT2 staining. (**i**) Cardiomyocytes from the same fibroblast cell line as DCM 1 that are reprogrammed using the non-integrating CoMiP (DCM 2), showing similar punctate, non-aligned TNNT2 staining. Scale bar, 200 μm

6. Pass through a 100 μm cell strainer into a 50 mL Falcon tube.
7. Count cells with Automated Cell Counter.
8. Dilute to 1×10^6 per mL.
9. Plate cells at 4×10^4 cells/cm^2 on Matrigel-coated 8-chamber coverslips.

3.8 Calcium Imaging

1. Wait for cells to begin contraction, around 3–5 days.
2. Treat with 5 μM Fluo-4 AM and 0.02 % Pluronic F-127 in Tyrode's solution for 5 min at 37 °C.

3. Wash with Tyrode's solution.
4. Conduct Ca^{2+} imaging using Carl Zeiss LSM 510 Meta confocal microscope with an oil immersion 63× objective (Plan-Apochromat 63×/1.40 Oil DIC M27) and analyze using Zen imaging software. Record spontaneous Ca^{2+} transients at 37 °C using a single-cell line scan mode (*see* **Note 5**) (Fig. 3). For analysis of the data, subtract extracellular background signal from calcium signals and normalize the calcium signal to the intracellular basal line (F_0). Transient amplitude is expressed as $\Delta F/F_0$. Decay Tau (mS) is calculated by mono-exponential curve fitting.

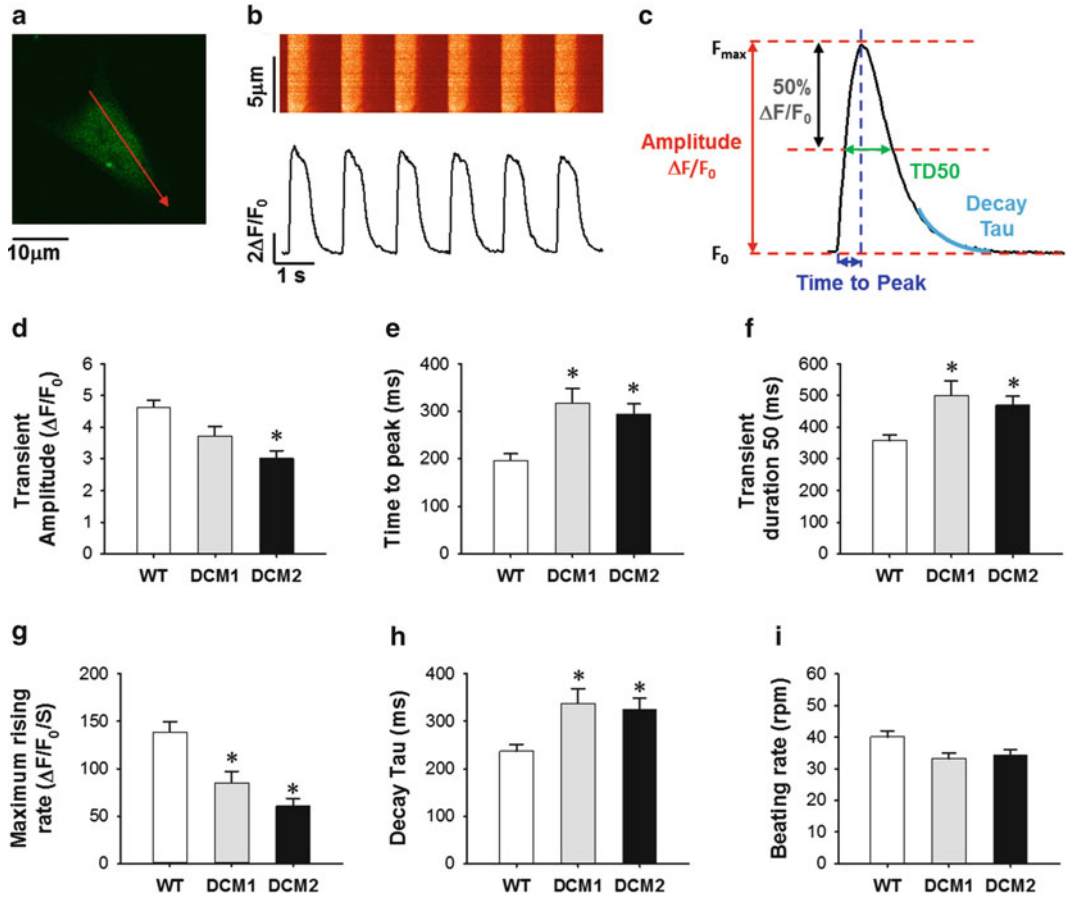

Fig. 3 Comparison of calcium handling properties between wild type (WT) and dilated cardiomyopathy (DCM) hiPSC-CMs demonstrating that lentiviral- and CoMiP-derived hiPSCs from the same DCM patient produce cardiomyocytes with similar functional abnormalities. (**a**) Line-select of time-lapse calcium imaging. (**b**) Representative recording of spontaneous calcium transient. (**c**) Measurement of calcium handling parameters from recording data. (**d–i**) Comparison of calcium transient amplitude, time to peak, transient duration 50, maximum rising rate, decay Tau, and beating rate among WT ($n = 53$), DCM1 ($n = 30$), and DCM2 ($n = 35$) hiPSC-CMs. *$P < 0.05$ vs. WT group by Student's *T*-test

4 Notes

1. Successful isolation should yield 10 vials of passage 2 fibroblasts suitable for reprogramming.
2. Typically, the hiPSC reprogramming process will take up to 1 month. After hiPSC colonies are obtained, they should be passaged up to 20 times before optimal differentiation can occur. This may take an additional 2 months. The cardiac differentiation process will produce beating cardiomyocytes after about 10 days.
3. For ideal differentiations, culture hiPSCs to at least passage 20. For most lines, there may be a passage "window" for optimal differentiation. This will allow for sustained, continuous, high-quality differentiations for many passages. This passage "window" likely varies from line to line. Continue passaging hiPSCs until high-efficiency differentiations (greater than 80 % cardiomyocytes) are obtained.
4. During cardiac differentiation, initial hiPSC monolayer confluency is a key factor. For most lines, hiPSC monolayers must be between 60 and 90 % confluent at the start of differentiation. This is somewhat variable from line to line, as lower or higher confluence may be required during differentiation. However, allowing hiPSCs to grow to full confluency during maintenance prior to cardiac differentiation is not recommended, as this may reduce the efficiency of differentiation.
5. Age of cardiomyocytes upon calcium imaging: cell characteristics may be dependent on maturation level or disease progression.

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Calcium Imaging in Pluripotent Stem Cell-Derived Cardiac Myocytes

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Abstract

The possibility to generate cardiomyocytes (CMs) from disease-specific induced pluripotent stem cells (iPSCs) is a powerful tool for the investigation of various cardiac diseases in vitro. The pathological course of various cardiac conditions, causatively heterogeneous, often converges into disturbed cellular Ca^{2+} cycling. The gigantic Ca^{2+} channel of the intracellular Ca^{2+} store of CMs, the ryanodine receptor type 2 (RyR2), controls Ca^{2+} release and therefore plays a crucial role in Ca^{2+} cycling of CMs. In the present protocol we describe ways to measure and analyze global as well as local cellular Ca^{2+} release events in CMs derived from a patient carrying a CPVT-causing RyR2 mutation.

Keywords: Cardiomyocytes, Calcium transients, Sparks, Confocal microscopy, Tachycardia

1 Introduction

Cardiovascular diseases (CVDs) are the most frequent cause of death in developed countries and their prevalence is expected to increase in future as population ages [1]. Approximately 43 % of CVDs are the consequence of ischemic cardiomyopathy and the resulting heart failure. However, in a smaller subset of mostly younger patients CVDs are caused by specific mutations in genes encoding cardiac ion channel subunits, cardiac structural proteins or their regulatory factors leading to various channelopathies (e.g., short QT syndrome, long QT syndrome, Brugada syndrome, catecholaminergic polymorphic ventricular tachycardia) and cardiomyopathies (e.g., dilated cardiomyopathy, hypertrophic cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy). Exploring pathophysiological mechanisms underlying these diseases and developing more effective therapeutic approaches has been hampered by lack of suitable human in vitro assay systems and shortcomings of existing cell-based and animal disease models [2–5]. CMs derived from disease-specific induced pluripotent stem cells (iPSCs) represent a novel in vitro cellular model of human inherited heart diseases with an unprecedented potential for generating new mechanistic insight into disease

pathophysiology and improving the process of drug development. The unique feature of this model is that it enables an unlimited supply of required disease-specific cell types and that the cell autonomous aspects of the disease and drug effects (therapeutic and toxic) can be studied on the genetic background of the affected patient [6]. However, the most important prerequisite for successful use of iPSC-derived CMs (iPS-CMs) for disease modelling, drug discovery, or regenerative medicine is that their functional and structural properties resemble those of adult CMs.

One of the essential features of CM physiology is their ability to rapidly increase cytosolic Ca^{2+} concentration in response to spontaneous or induced membrane depolarizations. In this process, small amounts of Ca^{2+} that enter into the cell through voltage-gated L-type Ca^{2+} channels (I_{Ca}) bind to ryanodine receptor type 2 (RyR2) in the membrane of the sarcoplasmic reticulum (SR) and in turn induce massive release of Ca^{2+} into the cytosol from intracellular Ca^{2+} stores in the SR. Sporadic subcellularly localized calcium releases can be observed in CMs during diastole as spatiotemporally restricted Ca^{2+} sparks [7]. They originate from a single RyR2 or a cluster of RYR2s and represent the elementary events of cardiac excitation–contraction (E-C) coupling. The recruitment and summation of many Ca^{2+} sparks in the beginning of systole produces global whole-cell Ca^{2+} transients. At this point of time the cytosolic Ca^{2+} concentration rises transiently approximately tenfold and activates Ca^{2+}-sensitive contractile protein troponin C. This is followed by induction of cross-bridge cycles at the molecular level, sarcomere shortening at the subcellular level and heart muscle contraction at the organ level. This process is terminated by removal of Ca^{2+} from the cytosol either out of the cell by the sodium–calcium exchanger (NCX) and plasma membrane calcium ATPase or back into the SR by the calcium ATPase located in the SR membrane (SERCA2a). Calcium homeostasis in CMs is perturbed in a number of acquired and inherited heart diseases, such as cardiac hypertrophy, heart failure, CPVT, and ARVC [8, 9]. Hence, analysis of calcium handling in CMs is an essential part in characterisation of their functional properties and identification of potential pathological features.

Analyses of Ca^{2+}-signalling parameters in cultured iPS-CMs demonstrated that I_{Ca}-gated SR Ca^{2+}-release is the primary mechanism for the release of Ca^{2+} when the cell is depolarized by the action potential [10]. Relaxation, in a manner similar to mammalian myocardium, was mediated by reuptake of Ca^{2+} into the SR and extrusion of Ca^{2+} by the NCX. Sporadic and brief Ca^{2+}-sparks and focal releases with spatiotemporal properties analogous to that of adult CM were also observed in human iPS-CMs [10, 11]. However, sparks were stochastic with a tendency of repetitive occurrence at the same site that may reflect an immature status of the human iPS-CMs [11]. However, more mature Ca^{2+} handling

properties could be induced in human embryonic stem cell (ESC)-derived CMs and iPS-CMs by prolonged cultivation [12], seeding of iPS-CMs onto microgrooved culture substrates [13] or electrical stimulation of 3D human iPS-CM-derived tissue [14]. These findings suggest that human ES-CMs and iPS-CMs represent a reliable Ca^{2+}-signalling model of adult CMs [15–18]. Moreover, iPSC-based in vitro models of human inherited cardiac arrhythmias and cardiomyopathies have demonstrated that disease-specific iPS-CMs are capable of reproducing the corresponding pathological calcium handling phenotype in vitro and that specific drugs ameliorate the electrical and Ca^{2+}-signalling disturbances in diseased CMs [10, 19–22].

Analyses of Ca^{2+}-signalling parameters in cultured CMs are traditionally based on the use of ratiometric calcium dyes such as Fluo-4 or genetically encoded calcium indicators such as GCaMP followed by the detection of spatiotemporal changes in their fluorescence intensities using epifluorescence or confocal microscopy [23, 24]. In this protocol we describe an experimental approach that can be used to investigate global and local cytosolic Ca^{2+} signals in healthy and diseased iPS-CMs in culture.

2 Materials

Solutions are prepared using ultrapure deionized H_2O (Milli-Q or alike, ≥18 MΩ·cm) and analytical grade reagents.

2.1 Ca^{2+} Imaging Solutions

1. HEPES-buffered, pyruvate containing Tyrode's solution [25]: For Ca^{2+} imaging, cells are kept in sterile-filtered (0.2 μm) Tyrode's solution containing (in mmol/l) NaCl 140, KCl 5.4, $MgCl_2$ 1, sodium pyruvate 2, $CaCl_2$ 1, glucose 10, HEPES 10, pH 7.4, adjusted with NaOH at room temperature (RT) (*see* **Note 1**).
2. Fluo-4 calcium indicator (Fluo-4, AM-ester, Life Technologies): A 1 mM Fluo-4 AM stock solution in DMSO is made by adding 46 μl DMSO into a 50 μg Fluo-4 AM vial (molecular mass of Fluo-4AM: 1096.95). The 1 mM stock can be aliquoted (5 μl aliquots, for instance) and kept at −20 °C for several months (PROTECTED FROM LIGHT). A working solution of 5 μM Fluo-4 AM is made by diluting the stock solution 1:200, e.g., adding 995 μl Tyrode's solution into the 5 μl stock solution (*see* **Note 2**). Prepare Fluo-4 working solutions on the day of experiment.
3. Caffeine (Sigma Chemicals): To prepare a 50 mM caffeine stock solution, 1 g caffeine is dissolved in 103 ml Tyrode's solution and is sterile filtered (0.2 μm). The stock solution is aliquoted in adequate volumes and stored at −20 °C. Working

solutions are prepared freshly on the day of the experiment. A typical caffeine working concentration for emptying SR Ca^{2+} stores of cardiomyocytes via RyR2 activation is 10 mM. However, this may not always be sufficient (*see* **Note 3**).

4. Ryanodine (Tocris, catalogue Nr. 1329; Molecular weight of Ryanodine: 493.55; solubility: 10 mM in ethanol, 25 mM in DMSO): 100 μl DMSO are added to the vial containing 1 mg lyophilized ryanodine, followed by vigorous vortexing. This will give a 20 mM ryanodine stock, which can be aliquoted and kept protected from light for 1–2 months at −20 °C. When used in μM to 1 mM concentration, ryanodine blocks the RyR2 Ca^{2+} release channels in the SR (*see* **Note 4**). For instance, a ≥200 μM working concentration of ryanodine will block the channels in a use dependent manner, i.e., the channel should be activated (typically by electrically pacing of CMs, if they do not beat spontaneously, as shown in Fig. 2) for several minutes. Do not use ryanodine concentrations <200 μM if you aim at a complete RyR2 channel block [26].
5. Isoproterenol (Isoprenaline, ISO; Sigma; MW of Isoprenaline hydrochloride: 247.72): A 10 mM ISO stock is made by dissolving 30 mg isoprenaline hydrochloride in 12.11 ml H_2O. Practically, a rough amount of ~20–30 mg isoprenaline hydrochloride is first transferred into a vial (e.g., a 15 ml sterile Falcon tube), and, according to the actual precise weight, the amount of 404 μl H_2O per mg isoprenaline hydrochloride is added into the vial to receive a 10 mM stock. The ISO stock solution is filtered (0.2 μm), aliquoted and kept at RT (<25 °C) for at least a week, protected from light. ISO solutions can be slightly yellow and become pink to brownish pink with time. In this case preparation of fresh solutions is recommended. The ISO working concentration is usually 1 μM. Dilution of the 10 mM stock into the working concentration can be done in two 1:100 steps in Tyrode's solution.

3 Methods

Cardiac differentiation of human iPSCs can be carried out by using different protocols, including the embryoid body-based method or small molecule-based directed differentiation protocol of monolayer iPSC cultures as described elsewhere [27–29] (Fig. 1). For Ca^{2+} imaging, microdissected beating clusters or monolayers of spontaneously beating iPS-CMs should be dissociated with collagenase B or Trypsin/EDTA at the desired day of differentiation. Single CMs are plated on 35 mm glass bottom dishes with 15 mm glass bottom diameter (P35G-1.5-14-C, MatTek, Ashland, MA) which have been coated with fibronectin (2.5 μg/ml) dissolved in

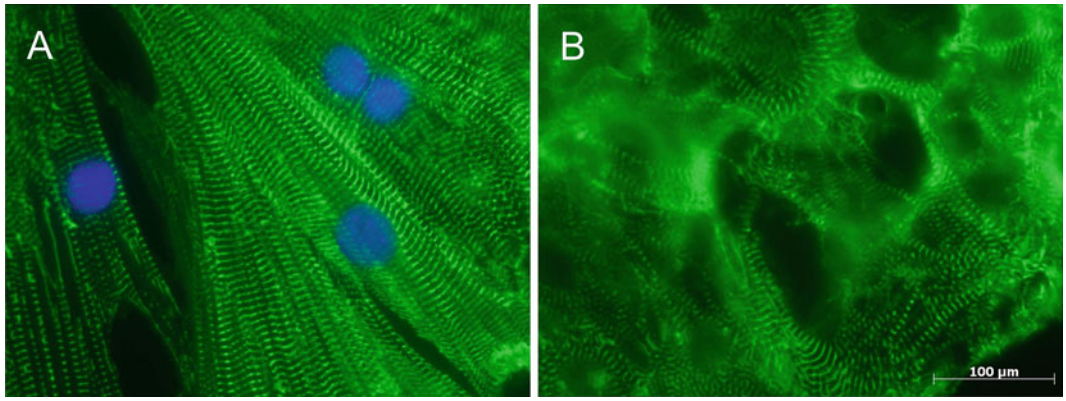

Fig. 1 Cross-striations in murine (**a**) and human (**b**) iPSC-derived CMs stained for a sarcomeric protein cardiac alpha-actinin. CMs derived from mouse iPS cells were prepared by methods as described previously [30]. This iPS cell line (clone AT25) was genetically modified to express puromycin resistance gene under the control of α-myosin heavy chain (α-MHC) promoter to enable purification of CMs. For the purpose of this analysis, murine iPS-CMs were analyzed on day 42 of differentiation. Purified human iPS-CMs (Cor.4U®) were kindly provided by Axiogenesis AG (http:/axiogenesis.com/) and plated on fibronectin-coated plates before staining

phosphate buffered saline (PBS, pH 7.4) for 3 h or overnight at 37 °C in a humidified atmosphere with 5 % CO_2. After plating, cells should be cultured for 36–72 h to allow attachment to fibronectin before use in Ca^{2+} imaging experiments.

3.1 Loading of Human iPS-CMs with the Fluo-4 Ca^{2+} Indicator

1. Take cells from the incubator and mount the culture dish stably onto the stage of the microscope.
2. Make the first microscopic overview using the transmitted light mode to ensure the presence and intactness of cells and to choose appropriate region for imaging (i.e., clear dish bottom, no debris, the appropriate cell density enabling imaging of single cells).
3. Aspirate the medium and wash the cells two times with 2 ml Tyrode's solution. Be careful not to remove too much solution during washing and make sure that cells are always covered with fluid (however, *see* **Note 5**).
4. After the second wash step, replace the Tyrode's solution with Tyrode's solution containing 5 µM Fluo-4 AM and leave the cells at RT for 20–30 min (*see* **Note 6**). During Fluo-4 loading cells should be kept in the dark and the dish covered with a lid to reduce evaporation.
5. Upon loading, wash the cells two times with Tyrode's solution and leave them for another 20 min at RT to complete de-esterification of Fluo-4 AM within the cells. The culture dish should be covered with a lid to reduce evaporation.

3.2 Microscopic Examination and Measurements

1. Upon Fluo-4 AM loading, select suitable CMs under the inverted fluorescence microscope. The criteria for cell selection are: appropriate Fluo-4 loading (*see* **Note 7**), cellular integrity (e.g., no blobs protruding from the cell), and spontaneous contractile activity. We typically perform this step of selection visually under the 20× oil immersion objective, under illumination with the mercury bulb, using the EGFP-filter set. Care is taken to accomplish this step rapidly and to avoid high illumination intensities.
2. Following the selection, move the cell of interest to the center in the field of view and switch the objective to 40× oil or 60× oil. If necessary, bring the cell back into the focus.
3. Switch off the visual inspection and record a first *x-y* scan under low laser intensity (0.1–1.0 % in our setup) using the 488 nm argon laser line (*see* **Note 8**). This first scan is an overview, allowing for selection of a region of interest (ROI) via the ROI tool of the measuring software. In most cases our ROIs are squares or rectangles, encompassing the selected cell.
4. Perform a brief fine tuning of the Z-focus to reach maximum fluorescence intensity. This is done by activating the continuous, fast *x-y* scanning mode and moving the focus knob up and down to find the focus position with the brightest fluorescence. To increase the signal intensity we sacrifice a bit of the confocality by opening the confocal aperture (c.a.) above the value recommended by the system for a particular objective (*see* **Note 9**). Further procedure and microscope settings depend on the type of measurement with regard to cellular Ca^{2+} signals.

3.3 Recording Global Cellular Ca^{2+} Transients

1. If global events, whole cell Ca^{2+} transients, have to be recorded, apply a fast *x-y* scan mode with reduced number of *x-y* pixels (e.g., a square ROI encompassing the cell is 250 × 250 or 125 × 125 pixels) (Fig. 2). However, if available, fast and sensitive cameras, instead of the photomultipliers we use in our setup, can also be used for this purpose. Please see details regarding our global Ca^{2+} transient recording settings in the legend of Fig. 2.
2. With the recording mode described in Fig. 2, the impact of toxic or pharmacological substances on Ca^{2+} transients can be investigated. This is shown in Fig. 3 for the effect of the RyR2 blocker ryanodine and the beta adrenergic agonist ISO, on spontaneous global Ca^{2+} transients of iPS cells. It is important to ensure a rapid and complete solution exchange while avoiding cell detachment and their subsequent loss. The optimal flow intensity during pipetting for solution changes is a matter of experience. However, to obtain an idea of the flow intensities during a solution change, one can visually control the cells

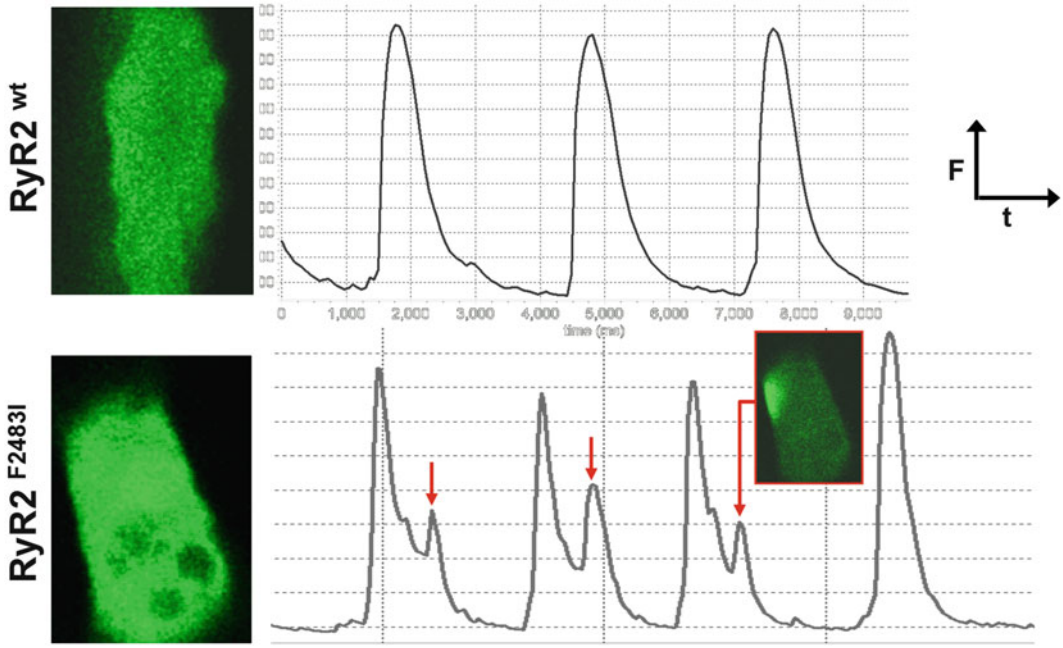

Fig. 2 Recording of global cellular Ca^{2+} transients in human iPS-CMs. Spontaneous global cellular Ca^{2+} transients of hiPSC-CMs expressing either wild type RyR2 (*top*) or the patient-derived CPVT-mutant $RyR2^{F2483I}$ (*bottom*), recorded under the fast *x-y* scanning mode of the FluoView1000 confocal microscope. Cells were loaded with 5 μM Fluo-4 and excited with the 488 nm argon line at 0.3 % laser intensity, emission was measured using the BA505IF long-pass filter. The photomultiplier voltage was set to 700 V, the confocal aperture was 350 μm. Under the 60× oil objective (numerical aperture of the objective: 1.35), square ROIs were chosen, encompassing the shown iPS-CM (note: only a *rectangular cut-out* of the original *square* ROIs is shown in the figure). The *square* ROIs had the dimensions of 256 × 256 pixels, corresponding to 33.4 × 33.4 μm (~130 nm/pixel). The sampling speed was 0.5 μs/pixel and 15.4 frames/s were recorded in the roundtrip-mode. Data were 12 bit digitized, no averaging was applied. With these settings, the spatial as well as the temporal resolution is sufficiently high to measure and compare global Ca^{2+} events. It also allows detection of local irregularities, as shown in the example of mutant cells heterozygous for $RyR2^{F2483I}$ mutation (see *inset* in the *bottom part* of the figure). This cell shows a locally restricted Ca^{2+} release that follows almost every global Ca^{2+} transient. In the context of the whole organ, the heart, such events could electrically destabilize the CMs and provoke arrhythmic events. The recorded data can be analyzed (upon background fluorescence intensity subtraction) in terms of amplitude, duration and frequency, for instance, using the same software which was used for recording (Olympus FluoView in our system). However, our software (and also the software of microscope systems from other companies) supports the export of the recorded transients into Excel for a more elaborate analysis

while he/she or a colleague is performing the pipetting. Practically, we are using the turbulences created during the solution change to mark the exact beginning of the solution change within the field of view. These turbulences are made visible during the measurement by activating, in addition to the regular channel used for recording Fluo-4 fluorescence, the transmitted light (i.e., transmission of 488 nm plus Fluo-4 emission)

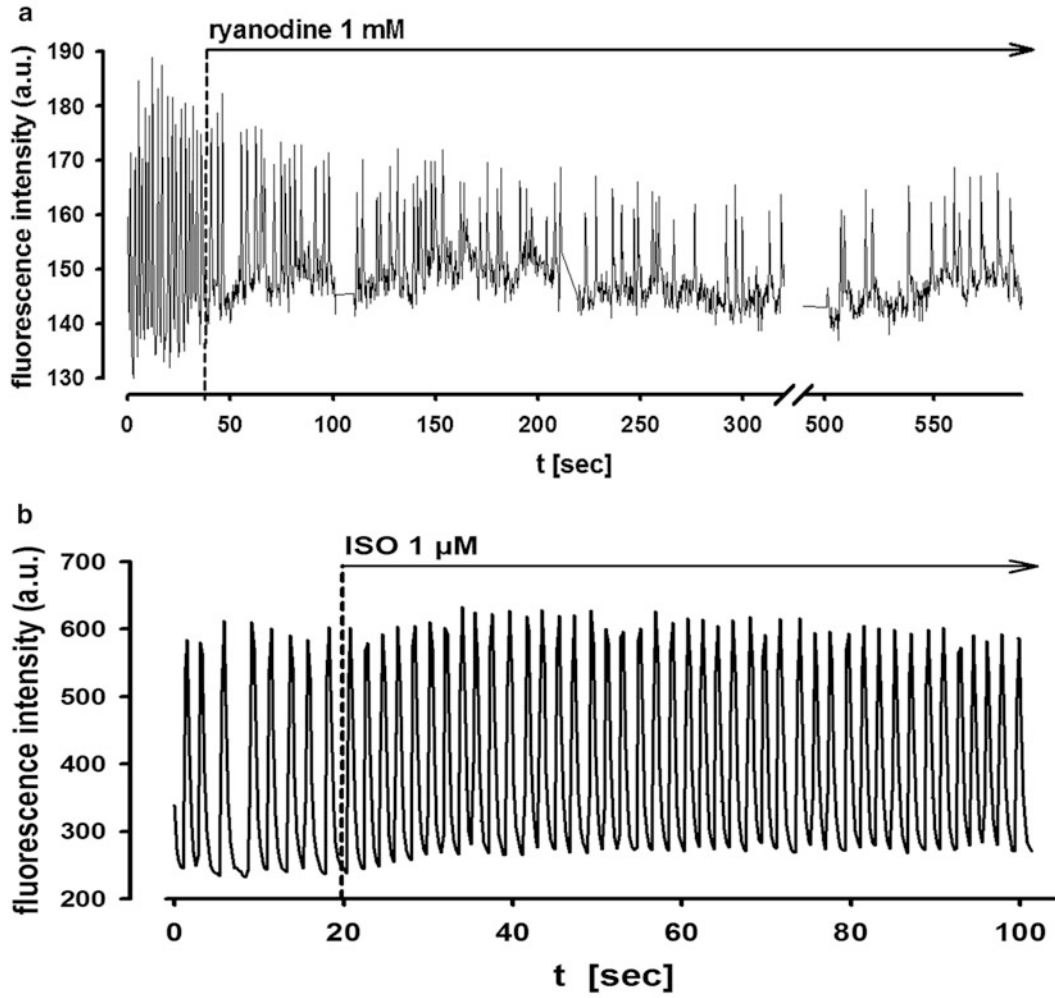

Fig. 3 The effect of 1 mM ryanodine (**a**) or 1 µM ISO (**b**) on spontaneous Ca^{2+} transients in human iPS-CMs. The experimental conditions are as described in Fig. 2. As can be seen in panel **a**, the addition of 1 mM ryanodine, a concentration high enough to completely block SR Ca^{2+} release via RyR2 (*see* **Note 4**), decreases the amplitude and frequency of Ca^{2+} transients, but does not completely abolish the spontaneous Ca^{2+} transients in iPS-CMs, even under prolonged ($\geq$10 min) incubation. The inability of ryanodine to completely block the Ca^{2+} transients is probably due to the presence of significant amounts of IP3 receptors, which can release Ca^{2+} from internal stores (for comparison see also Fig. 4a in ref. 18). As shown in panel **b**, the addition of 1 µM ISO has a modest effect on the frequency and virtually no effect on the amplitude of spontaneous Ca^{2+} transients in human iPS-CMs. In this example, the frequency was increased from eight transients per 20 s to ~10 transients per 20 s (however, compare to Fig. 4b in ref. 31)

channel in the DIC mode. However, the *x-y* mode of our microscope system is not fast enough to allow for appropriate recording and analysis of Ca^{2+} release events with faster kinetics, like fast Ca^{2+} waves and Ca^{2+} sparks. For this purpose, we use the line-scan mode described below.

3.4 Recording Local Ca^{2+} Release Events

1. For recording and analysis of local events, e.g., Ca^{2+} wavelets and Ca^{2+} sparks, operate the scanner in the line scan mode, i.e., it rapidly moves back and forth multiple times along the same trajectory, which has been defined before by the experimenter (e.g., between points a and b in Fig. 4). Thus, it rapidly reports changes in fluorescence intensity over time, along a defined, very narrow *x-y* cell slice. However, this highly repetitive event exposes the scanned region to photon stress and can often lead to a decrease in signal intensity due to fluorophore bleaching and/or to irreversible cell damage, often indicated by cessation of spontaneous beating and/or by cell contracture.
2. To decrease severity of these effects, operate the laser at the lowest possible intensity (often at 0.1–0.3 % setting of the 488 nm argon line). Depending on how frequently the recorded Ca^{2+} events occur, the duration of a particular line scan can be set from 2 s to >10 s, but a recording period longer than 10 s is not recommended. Figures 4 and 5 show examples of data which have been obtained under the line scan mode.
3. Adjust the focus manually to obtain maximum intensity read-out (try to do this as quickly as possible to prevent prolonged exposure of the cell to the laser).

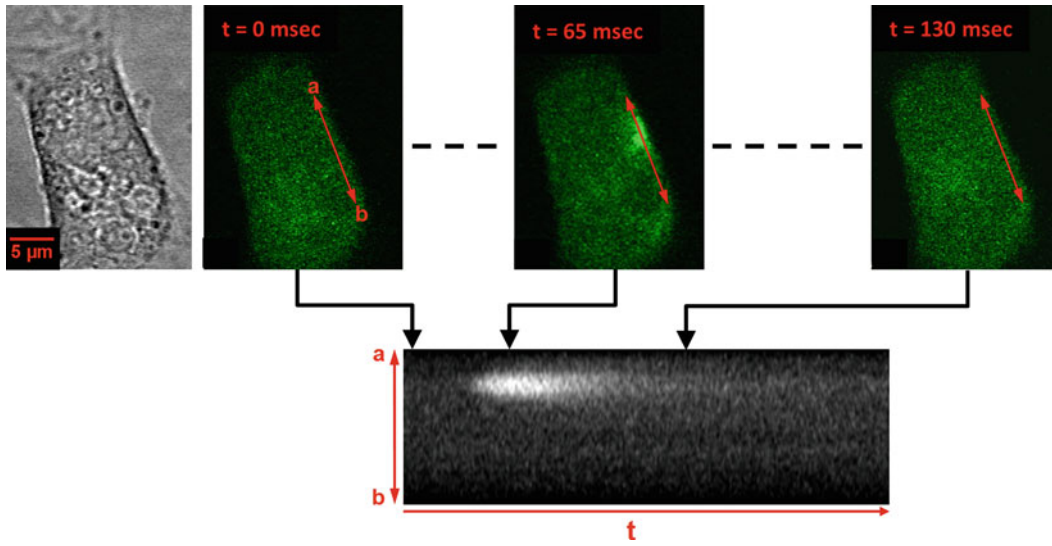

Fig. 4 Local Ca^{2+} release events recorded in the line scan mode. Shown is the same patient-derived iPS-CM heterozygously expressing the CPVT-mutant $RyR2^{F2483I}$ as in the *bottom panel* of Fig. 2. The *panel* on the *left* shows an intact and spontaneously beating (not perceptible in this representation) cell in *x-y* scan in the transmission mode under 60× oil. Fluorescence images in the *upper panel* show a cell after Fluo-4 loading, at different time points during diastole (from *left* to *right*: 0, 65 and 130 ms). The three ordinary *x-y* scans were recorded at different times, and are only shown to demonstrate the position of the scanned line relative to the cell and to demonstrate a spatially and temporary restricted Ca^{2+} release event (*see* the signal at $t = 65$ ms)

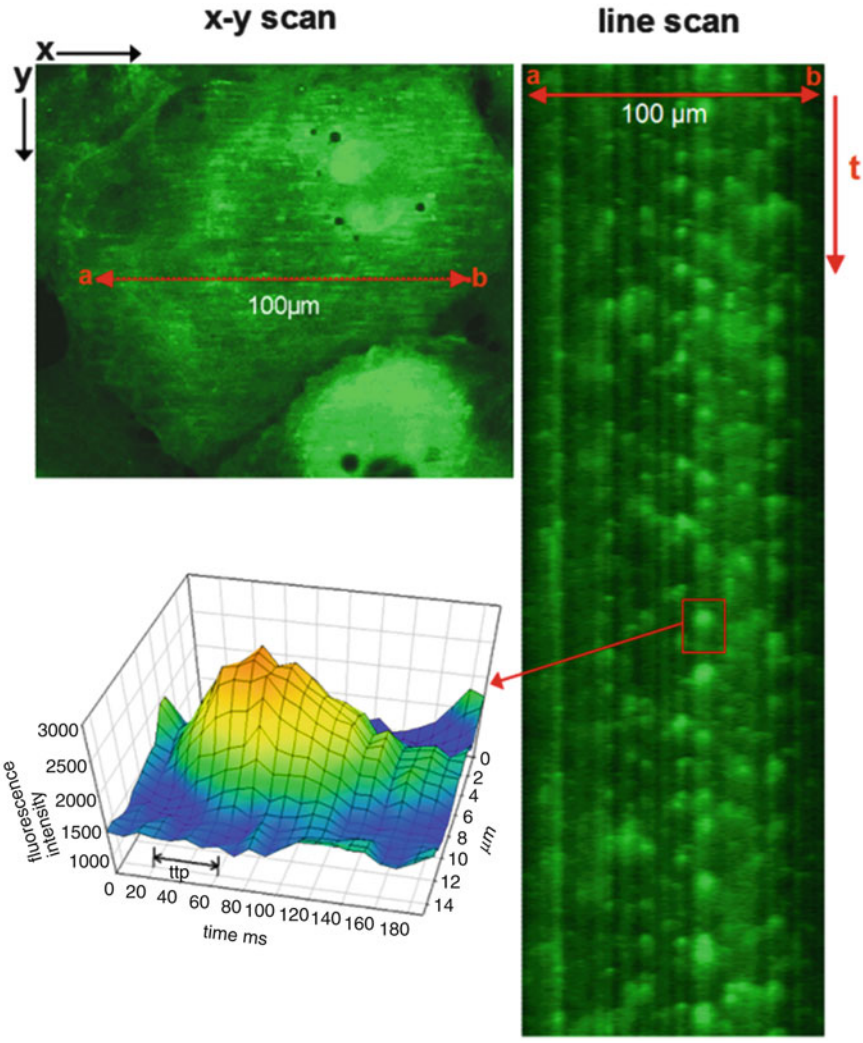

Fig. 5 Line scans enable resolving the frequency, the subcellular origin, and the time course of local Ca^{2+} release events. In this example, CPVT patient-derived iPS-CMs expressing mutant RyR2 ($RyR2^{F2483I}$), loaded with Fluo-4 have been used. CMs were from culture day 20. *Upper left panel*: regular *x-y* scan, showing the position of the line scan to be performed (*red line*). Note the presence of *multiple fluorescent stripes* along the *x* scanning direction. This "smear" in *x* direction reflects local Ca^{2+} release events, which are crossed by the scanner while performing the fast *x* part of an ordinary *x-y* scan. *a* and *b* mark the two points between which the line scan is performed (distance between points: 100 µm). *Right panel*: Scan along the line between points *a* and *b*. The scanned 100 µm correspond to 253 pixels (=414 nm/pixel under the chosen dimensions). Total recording time (t) was 5.89 s. Multiple local Ca^{2+} releases can be seen, the one highlighted by the *red rectangle* very likely appears repetitively from the same release site. Note that the release sites are not random and that most releases emerge repetitively from the same sites. *Lower left panel*: Graphical representation of the time course, spatial extension and Fluo-4 fluorescence intensity of a marked release event. Kinetic analysis involves, among other parameters, the time-to-peak value (ttp)

4. Then, perform a single *x-y* scan with a 256 × 256 pixel resolution. In the microscope software menu, switch to the line scan mode, and use the last *x-y* scan picture to draw a line along a distance *x* (from point a to point b), along which you want to

perform the line scan. This line is shown in red in Figs. 4 and 5. This line is usually in the range between 20 and 100 μm in length. Often, but not always, regions close to the edges of the cell, as in this example, are more likely to display local Ca^{2+} release events at high frequency.

5. Then, define how often the scanner has to measure the intensity along the defined line. In our system, to record local Ca^{2+} release over a distance x of ~35 μm and for a period t of 8 s, approximately 33,000 lines scans were performed. Thus, each pixel intensity along the line x can be recorded every 244 μs, i.e., 4/ms. This temporal resolution is high enough to record Ca^{2+} sparks, which typically last in differentiated CMs for about 40 ms (half time of fluorescence decay ~25 ms [32]).

3.5 Analysis of Recordings

1. With regard to analysis, one should consider that in contrast to ratiometric determination of Ca^{2+} absolute concentrations (such as for example with the Ca^{2+} indicator Fura), Fluo-4 imaging is, in most cases, not used to report absolute concentrations of $[Ca^{2+}]_i$. Rather, changes in $[Ca^{2+}]_i$ are recorded with Fluo-4 as changes (ΔF) of basal fluorescence intensity (F_0). Thus, already the non-stimulated cell will have a measurable Fluo-4 baseline fluorescence intensity F_0, which corresponds to a $[Ca^{2+}]_i$ of ~100 nM (*see* **Note 10**). To quantify a recorded Ca^{2+} event, the Fluo-4 intensity during this event, F, is related to F_0, in our lab with the quotient $(F - F_0)/F_0$. However, others often use the quotient F/F_0, the only difference being that the latter value is higher by a unity. Therefore, one should clearly describe how the fluorescence intensity data have been quantified.
2. An important factor in this respect is the background intensity. Care must be taken to remove the background reading of the photomultiplier (*see* **Note 11**). A mild data filter procedure (e.g., 3 × 3 pixel averaging), included in most confocal microscopy software, can be applied to decrease the unavoidable noise in fast measurements under low excitation intensity, without affecting kinetics of the recordings. However, one should always save the original data, i.e., still containing the background and not yet filtered.
3. The parameters we use most frequently for analysis of background-corrected and filtered, global and local Ca^{2+} transients are the maximum value for $(F - F_0)/F_0$, which is the amplitude, $(F_{max} - F_0)/F_0$, and the Full Duration at Half Maximum (FDHM).
4. There are several ways to calculate the amplitude and FDHM. One possibility is to use the interactive 3D surface plot plugin of ImageJ, open access graphic analysis software which can be downloaded from the NIH internet pages (http://imagej.nih.gov/ij/). For analysis, the original image is transferred to

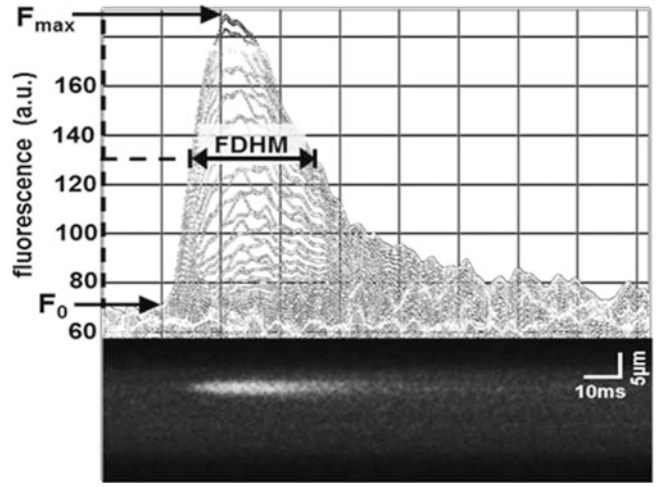

Fig. 6 An example for analysis of a local Ca^{2+} release event recorded in a human iPS-CM. CMs heterozygously expressing the CPVT mutant $RyR2^{F2483I}$ were loaded with 5 μM Fluo-4 and local Ca^{2+} release events recorded in the line scan mode. The local transient shown in the bottom *x-t* scan (=line scan) was recorded with a frequency of 4 lines/ms, was background-corrected and filtered by 3 × 3 pixel-average (Olympus FluoView software). The picture was then transferred to ImageJ and analyzed using the interactive 3D surface plot plugin of ImageJ. The visually determined values in the example shown in this figure are 190 arbitrary units (a.u.), 70 a.u. and 33 ms for F_{max}, F_0 and FDHM, respectively. The calculated amplitude, $(F_{max} - F_0)/F_0$, is calculated as (190 − 70)/70 and has the value of 1.7

ImageJ, e.g., by copying it to the clipboard of the operating system and pasting it into ImageJ. An example for the analysis of a local Ca^{2+} transient, recorded under the line scan mode, is shown in Fig. 6.

5. Pay attention to the following important aspects of analysis to ensure correctness of analysis and enable correct comparison of transients recorded from different iPS-CMs. First, the *x*-axis (= time scale) created by ImageJ does not have the same units (ms) as the original line scan, but can be easily converted into ms by relating ImageJ *x*-values to the duration (in ms) of the exported line scan segment. Second, the same settings should always be used in the ImageJ plugin when analyzing and comparing different data sets. Particularly, smoothing in the ImageJ analysis plugin should be kept as low as possible (not exceeding the value 10), and should be kept constant. After readout of F_{max}, F_0, and FDHM by the user, the plot should be saved.
6. Another possibility to analyze the transients is to export the line scan data to a spreadsheet application, such as Excel, where $(F_{max} - F_0)/F_0$ and FDHM can be calculated easily by applying a few custom made macros. For a more extensive analysis of local Ca^{2+} release recordings, especially for Ca^{2+} sparks, the

Bers lab developed a freely available ImageJ Plugin, SparkMaster [33]. In addition to amplitude and FDHM, the routine automatically also calculates FWHM, Full Width, Full Duration, Time-to-Peak, maximum steepness of spark upstroke, and the time constant of spark decay (for download please see: https://sites.google.com/site/sparkmasterhome/). For successful use of SparkMaster it is important to have data with an acceptable signal to noise ratio and that the appropriate criteria are defined (e.g., the minimum size of an event which should be recognized as spark and taken for analysis).

4 Notes

1. A 5× stock can be prepared and sterile filtered (0.2 μm) upon pH adjustment. The stock can be aliquoted as 5 ml aliquots in 50 ml sterile Falcon tubes and kept at ≤4 °C for several weeks. Before use of a 5 ml aliquot, always check for precipitates and floating material while shaking the tube. Discard and prepare fresh solution if necessary. For a 5× stock solution weight the following quantities of chemicals (in g/l): NaCl 40.908, $MgCl_2 \times 6H_2O$ 1.017, KCl 2.013, $CaCl_2 \times 2H_2O$ 1.470, D-glucose 9.010, sodium pyruvate 1.100, and HEPES 11.915.
2. Make sure to order the acetoxy-methyl-ester of Fluo-4 (Fluo-4 AM). The Fluo-4 ester will enter the cells and will be trapped inside them upon de-esterification. However, under prolonged duration of an experiment (>1–1.5 h after Fluo-4 loading) the Fluo-4 dye accumulation in subcellular compartments will become significant, visible as high-intensity fluorescent dots. This will increase the amount of background fluorescence with no or complex contribution to the Ca^{2+} signal.
3. Using 10 mM caffeine for SR Ca^{2+} store emptying might be sufficient for stationary caffeine concentrations, as used in experiments described in this protocol. The required caffeine concentrations may be considerably higher when applying caffeine via so-called Ca^{2+} puffs (see, for instance, ref. 18).
4. Low ryanodine concentrations, in the low μM to nM range [26], have partially opposite effects, i.e., RyR can be activated. Thus, although the supplier's (Tocris) product information sheet states that ryanodine is a potent inhibitor of Ca^{2+} release from SR, the IC_{50} values listed there for ryanodine, 10 and 2.8 nM for skeletal and cardiac RyR, respectively, refer to the high affinity, activating binding site(s) on the RyR. Thus, only ryanodine working concentrations >100 or 200 μM should be used to achieve complete RyR inhibition.
5. However, the glass bottom of the glass bottom dishes which is glued to the surrounding plastic bottom creates a reservoir for

medium/solution, preventing fast drying out of the cells. We measured the thickness of the plastic bottom, it amounts to ~0.47 mm. With a diameter of the glass bottom of ~13.3 mm, the volume of this reservoir is about 65 µl (assuming an even solution surface). Technically, this residual volume in the glass bottom is a dead volume, decreasing the nominal concentration of a substance (caffeine for instance) by ~6.1 % when the exchanged volume is 1 ml. During the wash steps, do not direct the flow of the rinsing solution towards the cells, since they will detach and will be lost.

6. Fluo-4 loading of cells can be enhanced by addition of the non-ionic detergent Pluronic® F-127 (Life Technologies): For that purpose, the Fluo-4 AM stock aliquot is mixed with an equal volume of 20 % (w/v) Pluronic in DMSO, followed by dilution into the measuring solution. However, we achieve satisfactory levels of Fluo-4 AM loading in the absence of Pluronic when using 5 µM working concentration of the Ca^{2+} indicator.
7. Under visual examination of cells using mercury bulb excitation and the GFP filter set, successful Fluo-4 loading will be indicated by green fluorescing cells with a more or less similar intensity. However, very bright cells should be excluded from measurements, because they are very likely damaged and have a Ca^{2+} overload.
8. Any illumination/filter setting which is suited for EGFP or FITC can be used. We recommend using a 505–525 nm band pass filter for measurements. However, if no other fluorophore besides Fluo-4 is used in the experiments, like, for instance, fluorescent membrane stain CellMask® orange, a 505 long pass filter can be used which will give a stronger signal.
9. We manually set a confocal aperture (c.a., or pinhole diameter) almost twice as high as the value recommended by the software for the 60× oil, i.e., from a c.a. of 105 µm to a c.a. between 150 and 300 µm, or up to 350 µm for thick cells. Higher values will also bring more out-of-focus, unrelated signal into the measurement and can be counterproductive. However, one has to determine her/his own compromise between intensity and spatial resolution.
10. For further helpful information on Fluo-4 properties (e.g., compartmentalization) and for an extensive comparison of various fluorescent Ca^{2+} indicators we recommend the ref. [34].
11. Part of the measured fluorescence intensity F upon illumination of cells with the 488 nm argon line will not originate from Fluo-4 emission but will be due to autofluorescence. This background can be significant and has to be removed, as described in the following example: let us assume that the background intensity is 200 (fluorescence intensity, arbitrary

units). If now a F_0 of 600 is measured and F_{max} of 1800, then, when the background is not subtracted (from both, F_0 and F_{max}), the quotient $(F_{max} - F_0)/F_0$ will amount to 2. However, when the background is subtracted, it will give the correct value of 4, which is twofold higher! The lower the Fluo-4 signal intensity, the higher this error will be. To correct for this unwanted contribution, in principle, one could determine the background intensity before loading the cells with Fluo-4 AM, by exciting the unloaded cell with the 488 nm argon line and recording the emission intensity. However, the actual argon laser intensity which will be used after Fluo-4 loading of the cells for the experiment is variable, and so will be the background intensity. Thus, we determine the background intensity for every measurement after performing the scans, by measuring the average intensity within a cell-free region and by subtracting this value from all pixels of the scan.

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Patient-Specific Induced Pluripotent Stem Cell Models: Generation and Characterization of Cardiac Cells

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Abstract

The generation of human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes has been of utmost interest for the study of cardiac development, cardiac disease modeling, and evaluation of cardiotoxic effects of novel candidate drugs. Several protocols have been developed to guide human stem cells toward the cardiogenic path. Pioneering work used serum to promote cardiogenesis; however, low cardiogenic throughputs, lack of chemical definition, and batch-to-batch variability of serum lots constituted a considerable impediment to the implementation of those protocols to large-scale cell biology. Further work focused on the manipulation of pathways that mouse genetics indicated to be fundamental in cardiac development to promote cardiac differentiation in stem cells. Although extremely elegant, those serum-free protocols involved the use of human recombinant cytokines that tend to be quite costly and which can also be variable between lots. The latest generation of cardiogenic protocols aimed for a more cost-effective and reproducible definition of the conditions driving cardiac differentiation, using small molecules to manipulate cardiogenic pathways overriding the need for cytokines. This chapter details methods based on currently available cardiac differentiation protocols for the generation and characterization of robust numbers of hiPSC-derived cardiomyocytes under chemically defined conditions.

Keywords: Human induced pluripotent stem cells, Cardiomyocyte, Cardiac differentiation, Cardiac assays, Small molecule, Wnt signaling pathway

1 Introduction

Since their establishment, human induced pluripotent stem cells (hiPSCs) have been considered a new gold standard for modeling human genetic-based diseases. Similar to human embryonic stem cells, hiPSCs have the potential to differentiate into virtually any cell type in the human body, offering enticing possibilities to study organ- and system-specific disorders, including diseases affecting the heart (1). Additionally, hiPSC-derived cardiomyocytes constitute a robust platform for the assessment of cardiotoxicity, a major contributor to the failure of potential novel drugs in later stages of clinical trials (2). Several genetic diseases bearing cardiac phenotypes have been modeled with hiPSCs including LEOPARD syndrome (3), long QT syndrome (4–7), Timothy syndrome (8), catecholaminergic polymorphic ventricular tachycardia (9–12),

familial dilated (13) and hypertrophic cardiomyopathies (14, 15), arrhythmogenic right ventricular cardiomyopathy (16–18), as well as an overlapping syndrome of a cardiac Na^+ channel disease (19). As the field has evolved, several differentiation protocols have been developed for the differentiation of hiPSCs toward the cardiac lineages (1). Currently, the most popular protocols rely on small-molecule-mediated temporal modulation of the Wnt pathway (20, 21). Here we present an adapted version of a small-molecule-based protocol, which has been used successfully across many independent hiPSC control and diseased lines.

2 Materials

2.1 Commercially Available Reagents

See Table 1.

Table 1
Commercially available reagents

Description	Manufacturer	Catalog number
Cell growth matrices		
Growth factor-reduced matrigel	Corning	354230
Laminin	Life Technologies	23017-015
Cell culture media		
DMEM/F12 with glutamine and HEPES	Life Technologies	11330-032
RPMI 1640 with L-glutamine	Life Technologies	11875093
DMEM with L-glutamine and without sodium pyruvate	Corning	10-017
M199 with L-glutamine	Corning	10-060
Medium supplements		
L-Ascorbic acid 2-phosphate	Sigma-Aldrich	A8960
Insulin	Life Technologies	12585-014
Transferrin	Sigma-Aldrich	T3705
Sodium selenite	Sigma-Aldrich	S5261
B-27 with insulin	Life Technologies	17504044
B-27 without insulin	Life Technologies	A1895601
Penicillin/streptomycin antibiotics (P/S)	Corning	30-002
Cytokines		
FGF2	PeproTech	100-18B
TGFβ	PeproTech	100-21
Buffers		
PBS without Ca^{2+} and Mg^{2+} (PBS−)	Corning	21-031
PBS with Ca^{2+} and Mg^{2+} (PBS+)	Corning	21-030
HBSS without Ca^{2+} and Mg^{2+} (HBSS−)	Corning	21-022
7.5 % sodium bicarbonate	Life Technologies	25080-094
0.5 M EDTA	Life Technologies	15575-020
BD Perm/Wash buffer	BD Biosciences	554723

(continued)

Table 1
(continued)

Description	Manufacturer	Catalog number
Cell dissociation reagents		
Accutase	Innovative Cell Technologies	AT 104
Collagenase type II	Worthington	LS004174
Taurine	Sigma-Aldrich	T8691-100G
EGTA	Sigma-Aldrich	E-4378-25G
25 % BSA	Life Technologies	A10008-01
Small molecules		
Y27632/Rock inhibitor ($Rock_i$)	Selleckchem	S1049
CHIR99021 (CH)	Selleckchem	S1263
IWP-2	Cayman Chemical	13951
Antibodies and staining reagents		
Donkey serum	Jackson ImmunoResearch	017-000-121
Mouse anti-sarcomeric alpha actinin	Sigma-Aldrich	A7811
Rabbit anti-NKX2.5	Santa Cruz	sc-14033
DyLight-488-conjugated donkey anti-mouse	Jackson ImmunoResearch	715-485-150
DyLight-549-conjugated donkey anti-rabbit	Jackson ImmunoResearch	711-505-152
R-phycoerythrin-conjugated donkey anti-mouse	Jackson ImmunoResearch	715-116-150
PerCP-conjugated donkey anti-rabbit	Jackson ImmunoResearch	711-126-152
Hoechst 33342 nuclear DNA stain	Life Technologies	H1399

2.2 Preparation of Extracellular Matrices (ECMs)

Add the corresponding volume of diluted ECMs to coat different tissue culture plates according to Table 2.

2.2.1 Growth Factor-Reduced Matrigel

Thaw a 10 ml vial of growth factor-reduced matrigel on ice for 1 hour (h) or overnight at 4 °C. Add 10 ml of cold DMEM/F12 medium and mix quickly and thoroughly. Quickly prepare 600 µl aliquots, to be stored at −80 °C.

2.2.2 Diluted Growth Factor-Reduced Matrigel

Thaw a 600 µl aliquot of growth factor-reduced matrigel on ice for 1 h or overnight at 4 °C. Add 50 ml of cold DMEM/F12 medium to a 50 ml falcon tube. Dilute the matrigel aliquot into DMEM/F12 and quickly mix thoroughly by inverting the falcon tube several times. Cell culture plates are subsequently incubated with matrigel solution to coat surfaces. Coated plates may be stored at 37 °C for up to 5 days prior to cell seeding.

2.2.3 Laminin

A 1 ml vial of laminin is thawed on ice and 50 µl aliquots are prepared for storage at −80 °C. For laminin coating, a 50 µl laminin aliquot is thawed on ice and diluted into a 5 ml solution of PBS+,

Table 2
Tissue culture plate formats and coating volumes

Tissue culture plate	ECM coating volume
6 cm^2 plate	3 ml
3.5 cm^2 plate	1.5 ml
12-well plate	0.5 ml/well
96-well plate	50 µl/well

mixed thoroughly and incubated on tissue culture dish surfaces overnight at 37 °C. Coated dishes may be stored at 4 °C for up to 7 days prior to cell seeding.

2.3 Cell Culture Medium

hiPSCs are maintained in Essential 8 (E8) medium (22). Prepare stock solutions and make aliquots as indicated in Table 3.

See **Note 1**.

2.3.1 hiPSC Culture Medium

Prepare a 500 ml bottle of E8 medium by adding supplements according to Table 4.

See **Note 2**.

2.3.2 Differentiation Initiation Medium: RPMI Medium with B27 Supplement Without Insulin (RB−)

Take a 500 ml bottle of RPMI and add one 10 ml vial of B27 without insulin. Add one 5 ml aliquot of P/S. Mix well. If the medium will be used within 1–10 days, then the bottle can be kept at 4 °C and 50 ml aliquots can be generated to be warmed when needed. Otherwise, 50 ml aliquots should be prepared for storage at −20 °C.

2.3.3 Differentiation Medium: RPMI Medium with B27 Containing Insulin (RB+)

Take a 500 ml bottle of RPMI and add one 10 ml vial of B27 with insulin. Add one 5 ml aliquot of P/S. Mix well. If the medium will be used within 1–10 days, then the bottle can be kept at 4 °C and 50 ml aliquots can be generated to be warmed when needed. Otherwise, 50 ml aliquots should be prepared for storage at −20 °C.

2.3.4 Maturation Medium: DMEM/M199 Medium with B27 Containing Insulin (D/199B+)

Take a fresh bottle of DMEM and remove 140 ml of the medium. Add 125 ml of M199. Add one 10 ml vial of B27 with insulin. Add one 5 ml aliquot of P/S. Mix well. If the medium will be used within 1–10 days, then the bottle can be kept at 4 °C and 50 ml aliquots can be generated to be warmed when needed. Otherwise, 50 ml aliquots should be prepared for storage at −20 °C.

Table 3
Stock solutions and aliquot volumes

Stock solution	Desired concentration	MW	Quantity needed	Resuspension volume	Aliquot volume (μl)
L-Ascorbic acid 2-phosphate	64 mg/ml	289.54	5 g	78.125 ml	500
Transferrin	53.5 mg/ml	N/A	1 g	18.692 ml	100
Sodium selenite	700 μg/ml	172.94	35 mg	50 ml	10
FGF2	100 μg/ml	N/A	1 mg	10 ml	500
TGFβ1	100 μg/ml	N/A	100 μg	1 ml	10

Table 4
Preparation of E8 medium

Component	Stock solution	Take from stock	Final concentration
DMEM/F12 with glutamine and HEPES	1×	500 ml	1×
Sodium bicarbonate	75 mg/ml	3.62 ml	543 μg/ml
L-Ascorbic acid 2-phosphate	64 mg/ml	500 μl	64 μg/ml
Insulin	4 mg/ml	2.5 μl	20 μg/ml
Transferrin	53.5 mg/ml	100 μl	10.7 μg/ml
Sodium selenite	700 μg/ml	10 μl	14 ng/ml
FGF2	100 μg/ml	500 μl	100 ng/ml
TGFβ	100 μg/ml	10 μl	2 ng/ml
Pen/strep	100×	5 ml	1×

2.4 Small Molecules

2.4.1 Rock Inhibitor/ Y27632: $Rock_i$

For a 10 mM (2,000×) solution, re-suspend 10 mg vial in 3.122 ml of DMSO. Mix well, vortex, and make 25–50 μl aliquots, to be stored at −20 °C. Aliquots may be reused up to three times.

2.4.2 CHIR99021: CH

For a 12 mM (2,000×) solution, re-suspend 25 mg vial in 4.152 ml of DMSO. Mix well, vortex, and make 25–50 μl aliquots, to be stored at −20 °C. Aliquots may be reused up to three times.

2.4.3 IWP-2

For a 5 mM (1,000×) solution, re-suspend 10 mg vial in 4.286 ml of pre-warmed DMSO. Mix well, vortex, and make 25–50 μl aliquots, to be stored at −20 °C. Aliquots may be reused up to three times.

2.5 Dissociation Reagents

Add 500 μl of 0.5 M EDTA to a bottle of PBS−.

2.5.1 hiPSC Dissociation Solution: 0.5 mM EDTA

2.5.2 Collagenase Type II Solution

Cardiomyocytes are dissociated in a solution containing 200 units/ml of HBSS−. Estimate the required volume of collagenase II solution, and calculate and weigh the mass necessary to reach the target concentration in units/ml. Dissolve into HBSS− and filter sterilize. The solution can be kept at 4 °C for up to 1 week.

2.5.3 Cardiomyocyte Dissociation Solution

Prepare cardiomyocyte dissociation solution immediately before use. For 1 ml cardiomyocyte dissociation solution, prepare the stock solutions in tissue culture-grade water as described in Table 5.

2.6 Buffers

2.6.1 Immunofluorescence Permeabilization Buffer

In order to prepare 50 ml of immunofluorescence permeabilization buffer, dilute 100 μl of Triton-X in 50 ml of PBS+, for a final concentration of 0.2 % Triton-X.

2.6.2 Immunofluorescence Dilution Buffer

In order to prepare 50 ml of immunofluorescence buffer, prepare the stock solutions listed in Table 6 and add the appropriate volumes for the final desired concentrations.

Table 5
Preparation of cardiomyocyte dissociation solution

Component	Stock solution	Take from stock (μl)	Final concentration
Collagenase II solution	200 units/ml	880	200 units/ml
Taurine	200 mM	100	1 mM
EGTA	0.2 mM	10	0.1 mM
BSA	250 mg/ml	8	1 mg/ml

Table 6
Preparation of immunofluorescence dilution buffer

Component	Stock solution	Take from stock	Final concentration
Milli-Q water	N/A	42.13 ml	N/A
Tris base, pH 7.5	1 M	1 ml	20 mM
NaCl	2 M	3.87 ml	155 mM
EGTA	50 mM	2 ml	2 mM
$MgCl_2$	100 mM	1 ml	100 mM

2.6.3 Immunofluorescence Blocking Buffer

In order to prepare 10 ml of immunofluorescence blocking buffer, take 9.5 ml of immunofluorescence dilution buffer and add 500 μl of donkey serum for a final concentration of 5 %.

2.6.4 FACS Dilution Buffer

BD Perm/Wash buffer is sold as a 10× concentrate that must be diluted in PBS− before use. For 10 ml of 1× ready-to-use BD Perm/Wash buffer, add 1 ml of 10× concentrate BD Perm/Wash buffer to 9 ml of PBS−.

2.6.5 FACS Blocking Buffer

For 10 ml of FACS blocking buffer, take 9.5 ml of 1× ready-to-use BD Perm/Wash buffer and add 500 μl of donkey serum for a final concentration of 5 %.

3 Methods

3.1 hiPSC Culture

hiPSCs are grown as confluent monolayers on growth factor-reduced matrigel-coated 6 cm^2 plates. Passaging is performed at a ratio of 1:4–1:10 every 4–6 days and achieved as outlined below:

1. Wash cells once with warm PBS−.
2. Add 2 ml of warm 0.5 mM EDTA in PBS−.
3. Incubate for 5 min at 37 °C.
4. Aspirate the 0.5 mM EDTA and re-suspend cells in 4 ml of E8 medium. If cells do not detach completely, use a cell lifter to gently scrap them and break larger clusters by pipetting 6–10 times.
5. Split cells as required and add $Rock_i$ to a final concentration of 5 μM.

3.2 hiPSC Seeding for Cardiac Differentiation

Cardiac differentiation is typically performed in a 12-well plate format, although other formats can be used with the appropriate adjustments to cell growth surfaces. Prior to seeding, coat a 12-well plate with diluted growth factor-reduced matrigel as described in Sections 2.2 and 2.2.1.

1. When hiPSCs (6 cm plate) reach 80–90 % confluence, aspirate E8 medium and wash cells once with 4 ml of warm PBS−.
2. Add 1.5 ml of warm Accutase, and move the plate in perpendicular directions to spread the enzyme evenly. Remove 1 ml of the Accutase and move the plate in perpendicular directions again.
3. Incubate hiPSC for 3–5 min at room temperature, until cells are singularized. *See* **Note 3**.
4. Remove Accutase quickly and add 4 ml of maintenance medium. Pipette up and down 6–10 times to ensure that cells

are singularized or reduced to very small clusters. Transfer cells to a 15 ml Falcon tube. Add 5 μM of $Rock_i$ (2.5 μl of a 10 mM aliquot).

5. Count cells in the suspension.
6. Prepare 12.5 ml of a second suspension containing 5 μM $Rock_i$ in which the cell concentration is adequate for the desired number of cells to be plated in each well. *See* **Note 4**.
7. Plate 1 ml of the cell suspension prepared in step 4 in each well of the 12-well plate. Move the plate vigorously in perpendicular directions to ensure that cells are evenly distributed.

3.3 Cardiac Differentiation

The protocol described herein is an adaptation of a previously described small-molecule-based method (20, 23).

The key differences are the following:

1. Washing hiPSCs before cardiac differentiation/induction. The purpose of this step is to remove traces of FGF at the beginning of differentiation.
2. Use of insulin during CH treatment. We have observed that cell death can significantly impact cardiac differentiation. Insulin, acting as a pro-survival cytokine, facilitates survival during the first 48 h of the cardiac differentiation protocol.
3. Washing hiPSCs after cardiac differentiation/induction. Insulin has been proposed to have a detrimental effect on cardiac differentiation after 72–96 h of induction (24). Therefore, cultures are washed with RPMI before the addition of RB−.
4. Switching to D/199B+ after day 30 of cardiac differentiation. After cardiomyocytes are differentiated, it is recommended that they are maintained in culture for an additional time to allow for further maturation. The nutrient composition of RPMI medium is relatively conservative; thus, cardiomyocyte death can occur also at later stages of differentiation/maturation due to stringent culture conditions. Thus, an adaptation of the medium used for neonate mouse cardiomyocyte cultures has been incorporated in this protocol to promote an environment richer in nutrients, allowing for enhanced cardiomyocyte health and survival during maturation.

3.3.1 Day 0: Induction

1. When hiPSCs plated on the 12-well plate reach 100 % confluence (within 2–5 days of plating), warm an aliquot of RPMI and one of RB+ medium.
2. Take 24.5 ml of RB+ and add CH to a final concentration of 6 μM. Mix well. *See* **Note 5**.
3. Wash cells once with warm RPMI medium (1 ml per well).
4. Add 2 ml of RB+ with CH to each well. Record the time at which CH is added.

3.3.2 Day 2: Wash and Switch to RB–

After CH treatment, mild to considerable cell death is expected. Proceed with the protocol as outlined below:

1. Warm an aliquot of RPMI and RB− medium.
2. 48 h after adding CH to the cells:
   (a) Wash cells once with warm RPMI medium (2 ml per well).
   (b) Add 2 ml of RB− to each well.

3.3.3 Day 4: IWP-2 in Conditioned Medium

1. Warm an aliquot of RB− medium.
2. Collect 1 ml of conditioned medium from each well of the differentiation plate at 96 h after adding CH, plus an excess of 0.5 ml, and transfer to a 50 ml Falcon tube. The total volume of conditioned medium collected must be 12.5 ml.
3. Add equal volumes (12.5 ml) of RB−. Add IWP-2 to the conditioned medium to a final concentration of 5 μM. Mix well. *See* **Note 6**.
4. Aspirate the remaining conditioned medium from all wells of the differentiation plate.
5. Add 2 ml of combined medium with IWP-2 to each well.

3.3.4 Day 6: Medium Change

See **Note 7**.

1. Warm an aliquot of RB− medium.
2. 144 h after adding CH to the cells: remove medium and add 2 ml of fresh RB− to each well.

3.3.5 Day 8: Medium Change

1. Warm an aliquot of RB− medium.
2. 192 h after adding CH to the cells: remove medium and add 2 ml of fresh RB− to each well.

3.3.6 Day 10: Medium Change (Switch to RB+)

See **Note 8**.

1. Warm an aliquot of RB+ medium.
2. 240 h after adding CH to the cells: remove medium and add 2 ml of fresh RB+ to each well.

3.3.7 Day 11–30: Differentiation and Maintenance

Change medium every 2–3 days with RB+ until day 30. *See* **Note 9**.

3.3.8 Day 31–45+: Maturation

Switch medium to D/199B+:

1. Warm an aliquot of D/199B+ medium.
2. Remove medium from cells and add 2 ml of fresh D/199B+ to each well.
3. Change medium every 2–3 days with D/199B+ until cardiomyocytes have achieved desired time points.

   See **Note 10**.

3.4 Characterization of hiPSC-Derived Cardiomyocytes

Bona fide hiPSC-derived cardiomyocytes express appreciable levels of the contractile protein sarcomeric α-actinin and the transcription factor NKX2.5. Co-staining of cells with this combination of cardiac markers robustly identifies hiPSC-derived cardiomyocytes.

3.4.1 Cardiomyocyte Dissociation

Beating clusters or sheets of hiPSC-derived cardiomyocytes can be microdissected, dissociated, and replated for specific downstream applications. *See* **Note 11**.

1. Wash cells once with warm HBSS−.
2. Add 1 ml of collagenase II solution. Add 10 μM $Rock_i$ and incubate for 30 min at 37 °C, swirling the plate or vortexing the microdissected cells every 5 min.
3. Pipette cells repeatedly 5–10 times to break larger clusters and transfer cells to a 2 ml Eppendorf tube.
4. Add 1 ml of dissociation solution dropwise. Mix gently.
5. Pass cells through a 20 G syringe needle 3–6 times until larger clumps can no longer be observed by the eye.
6. Spin cells for 5 min at 130 × *g* and re-suspend cells in 1 ml of D/199B+. Add 10 μM $Rock_i$.
7. Count cells and replate at an appropriate density. For a 3.5 cm plate, a minimum of 2×10^5 cells/plate should be added. Add 10 μM $Rock_i$.

   See **Note 12**.

3.4.2 Flow Cytometry

Flow cytometry allows for robust, quantitative, and rapid estimation of cardiomyocyte throughputs in a given differentiation experiment. The staining protocol outlined below relies on co-staining of undifferentiated hiPSCs (negative control) and hiPSC-derived cardiomyocytes resulting from a differentiation experiment with sarcomeric α-actinin and NKX2.5. *See* **Note 13**.

1. Dissociate cardiomyocytes, and following resuspension, strain cells through a 100 μM nylon mesh.
2. Count cells and prepare a suspension of 1×10^6 cells/ml.
3. Take 2×10^5 cells (200 μl of suspension at 1×10^6 cells/ml) and transfer to an Eppendorf tube.
4. Centrifuge the cell suspension at 200 × *g* in for 2 min. Discard supernatant by carefully aspirating with a micropipette to avoid losing cells.
5. Re-suspend cells in 100 μl of 1× BD Perm/Wash buffer and incubate for 10 min at room temperature (RT).
6. Centrifuge the cell suspension at 200 × *g* for 2 min. Discard supernatant.
7. Re-suspend cells in 100 μl of FACS blocking solution and incubate for 15 min at RT.

8. Centrifuge the cell suspension at 200 × *g* for 2 min. Discard supernatant.
9. Re-suspend cells in 100 µl of primary antibody mastermix that contains mouse anti-sarcomeric α-actinin (1:100) and rabbit anti-NKX2.5 (1:100) in blocking solution. Incubate for 30 min at RT.
10. Centrifuge the cell suspension at 200 × *g* for 2 min. Discard supernatant.
11. Wash once with 100 µl of 1× BD Perm/Wash buffer. Spin at 800 rpm for 2 min. Discard supernatant.
12. Re-suspend in 100 µl of secondary antibody mastermix that contains phycoerythrin-conjugated donkey anti-mouse (1:100) and PerCP-conjugated donkey anti-rabbit (1:100) in FACS blocking solution. Incubate for 30 min at RT in the dark.
13. Centrifuge the cell suspension at 200 × *g* for 2 min. Discard supernatant.
14. Wash once with 100 µl of 1× BD Perm/Wash buffer.
15. Centrifuge the cell suspension at 200 × *g* for 2 min. Discard supernatant.
16. Re-suspend cells in 200 µl of PBS−. Keep cells on ice in the dark until ready to analyze.

Expected Results

Figure 1 exemplifies FACS analysis of NKX2.5 and sarcomeric α-actinin results from hiPSC before and after cardiac differentiation. Cellular debris and cell doublets were eliminated, as well as

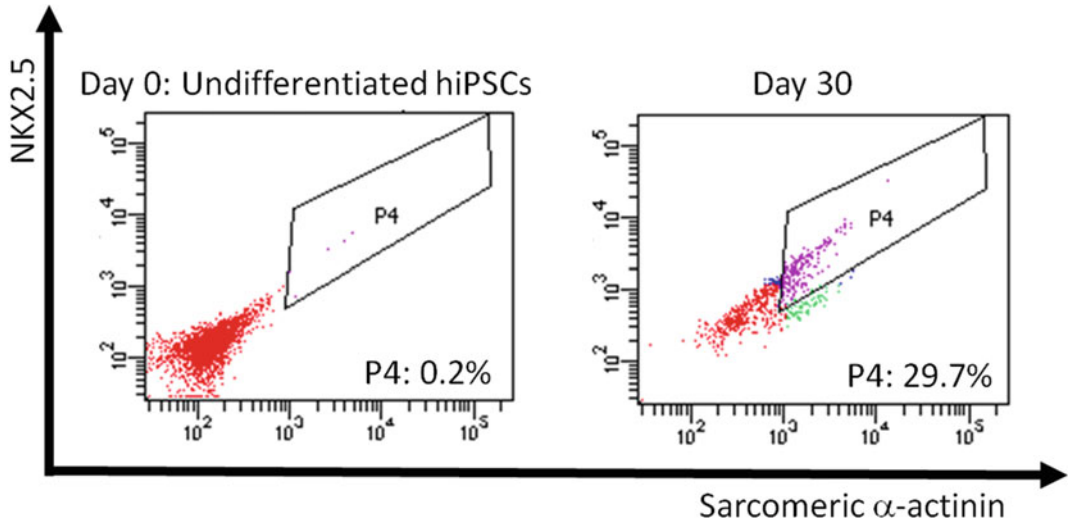

Fig. 1 An example of FACS analysis of sarcomeric α-actinin and NKX2.5 coexpression as an indicator of cardiomyocyte throughput. Gate P4 highlights double-positive cells in undifferentiated cells on day 0 and hiPSC-derived cardiomyocytes on day 30

individual signals for both antibodies were analyzed through gates not shown here. Gate P4 was set after analysis of stained undifferentiated hiPSC and adjustment of the negative control signals to the first three decades of each axis.

3.4.3 Immunofluorescence Microscopy

Cardiomyocytes may be dissociated and replated on laminin-coated dishes that are appropriate for cell imaging such as chamber slides, glass coverslips, and black-walled optic plates for immunofluorescence-based microscopy analysis.

1. Wash cells once with PBS+ solution.
2. Fix cells with 4 % PFA for 30 min at RT.
3. Wash cells once with PBS+ solution.
4. Permeabilize cells with immunofluorescence permeabilization buffer for 10 min at RT.
5. Block cells with immunofluorescence blocking buffer for 30 min at room temperature.
6. Prepare primary antibodies in immunofluorescence blocking solution:

   Mouse anti-sarcomeric α-actinin (1:100).

   Rabbit anti-NKX 2.5 (1:100).

   Aspirate blocking solution and add primary antibody mixture. Incubate overnight at 4 °C in a humidified chamber.
7. Wash cells three times for 5 min at RT with PBS+.
8. Prepare secondary antibodies and DNA stain in immunofluorescence blocking solution:

   DyLight-488-conjugated donkey anti-mouse (1:100).

   DyLight-549-conjugated donkey anti-rabbit (1:100).

   Hoechst 33342: 1 μg/ml.

   Aspirate PBS+ and add secondary antibody mixture. Incubate for 2 h in the dark at RT in a humidified chamber.
9. Wash cells three times for 5 min at RT with PBS+.
10. Mount cells and maintain at 4 °C in the dark until ready to image by using confocal microscopy.

Expected Results

Figure 2 exemplifies results obtained from immunofluorescence staining analysis of sarcomeric α-actinin and NKX2.5 in hiPSC following cardiac differentiation. Cells are considered positive for sarcomeric α-actinin when a clear striated pattern can be visualized. Cardiomyocytes are also defined by expression of NKX2.5, which is found confined to the cell nucleus.

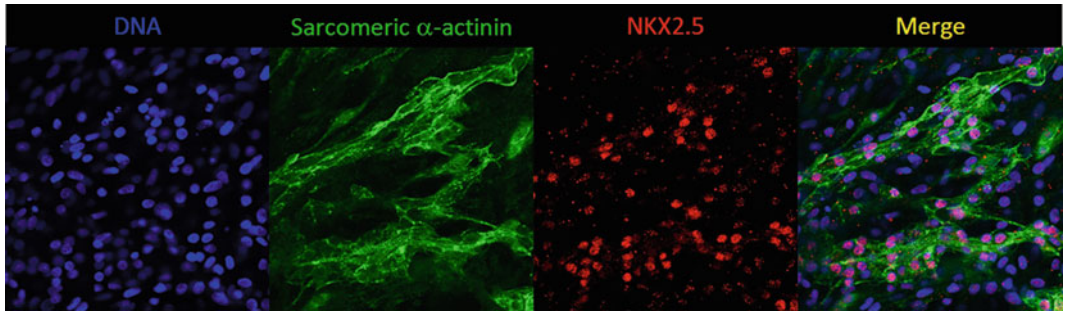

Fig. 2 An example of immunofluorescence analysis of sarcomeric α-actinin and NKX2.5 coexpression on day 30 of differentiation as an indicator of cardiomyocyte throughput

4 Notes

1. The solvent for all stock solutions is tissue culture-grade water, except for TGFβ, for which 10 mM citric acid with pH = 3 should be used. When preparing the ascorbic acid stock solution, pre-warm, pre-warm tissue culture-grade water to 37 °C before slowly dissolving with constant agitation and vortexing. Crystals may take several hours to dissolve completely. Store all aliquots of stock solutions at −20 °C.
2. The cytokines in E8 medium are not stable at 37 °C for long periods of time. Therefore it is preferable to warm the medium to room temperature instead of 37 °C.
3. Since Accutase is an aggressive enzyme, cell dissociation should be monitored frequently as some cell lines will singularize and even detach at a shorter time. If it is noted that cells start to peel off, remove the enzyme immediately. If all cells become detached, add 3 ml of maintenance medium, pipette up and down to collect all cells, and transfer to a 15 ml Falcon tube. Add 5 μM of $Rock_i$ (2.5 μl of a 10 mM aliquot) and spin at 800 rpm for 5 min. Re-suspend cells in 4 ml of maintenance medium, add 5 μM of $Rock_i$, and proceed to next step (4).
4. Seed 1×10^5–5×10^5 cells per well in matrigel-coated 12-well plates. Cell density should be optimized for each cell line, so confluence is reached within 2–5 days after plating. Ideally, cells should reach confluency within wells at the same approximate time frame that it took to reach confluency in 6 cm plates.
5. CH aliquots are prepared at 12 mM (2,000×), so for 24.5 ml of RB+, 12.25 μl of a CH aliquot is required. Also note that 6 μM CH for 48 h seems to be optimal for most cell lines; however, specific lines may have different requirements. It is recommended that a range of 6–14 μM is tested at treatment durations of 24 h (12–14 μM) and 48 h (6–8 μM).

6. IWP-2 aliquots are prepared at 5 mM (1,000×), so if a whole 12-well plate is used, add 25 μl of IWP to the 25 ml of combined medium prepared in step 3.
7. From this time point onward, cells may display a type of growth pattern that resembles epithelial-to-mesenchymal transition (EMT). As a result, instead of growing flat and spreading out horizontally, they may grow vertically and pile up in the form of foci in the well. This type of growth is indicative of successful mesoderm and precardiac mesoderm formation.
8. If medium is changed every 2 days, add 2 ml of medium per well. If medium is changed every 3 days, add 3 ml of medium per well.
9. At this time point, there should be sheets and/or clusters of beating cells that can be readily visualized by light microscopy. Beating is first observed between days 7 and 11 for most of cell lines.
10. If medium is changed every 2 days, add 2 ml of medium per well. If medium is changed every 3 days, add 3 ml of medium per well.
11. 24 h before dissociation, tissue culture dishes should be coated with 10 μg/ml laminin in PBS+, overnight at 37 °C.
12. Dissociated cardiomyocytes may take up to 3–5 days to start beating again. Change medium every 2–3 days.
13. Cardiomyocytes can display considerable autofluorescence within the 488 nm wavelengths; thus, secondary antibodies with emissions within the yellow and far-red regions of the light spectrum are preferable.

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Differentiation of Human Pluripotent Stem Cells to Cardiomyocytes Under Defined Conditions

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Abstract

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) can differentiate to cardiomyocytes in vitro, offering unique opportunities to investigate cardiac development and disease as well as providing a platform to perform drug and toxicity tests. Initial cardiac differentiation methods were based on either inductive co-culture or aggregation as embryoid bodies, often in the presence of fetal calf serum. More recently, monolayer differentiation protocols have evolved as feasible alternatives and are often performed in completely defined culture medium and substrates. Thus, our ability to efficiently and reproducibly generate cardiomyocytes from multiple different hESC and hiPSC lines has improved significantly.

We have developed a directed differentiation monolayer protocol that can be used to generate cultures comprising ~50 % cardiomyocytes, in which both the culture of the undifferentiated human pluripotent stem cells (hPSCs) and the differentiation procedure itself are defined and serum-free. The differentiation method is also effective for hPSCs maintained in other culture systems. In this chapter, we outline the differentiation protocol and describe methods to assess cardiac differentiation efficiency as well as to identify and quantify the yield of cardiomyocytes.

Keywords: Human embryonic stem cells, Human induced pluripotent stem cells, Cardiac differentiation, Cardiomyocyte characterization

1 Introduction

The differentiation of human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) into cardiomyocytes has provided exciting opportunities to examine these cells which otherwise can only be obtained through invasive biopsies of patients. These cardiomyocytes can be used not only to study heart development and diseases but also to perform drug and toxicity testing (1). This type of safety pharmacology is important because the heart is remarkably sensitive to the side effects of drugs and other compounds. The generation of hiPSCs from patients with genetic cardiac diseases also allows for the production of cardiomyocytes that capture the genome of the individual, offering the prospect to model these diseases in vitro (2, 3). These patient-specific cells

can be used to study the mechanisms underlying the disease pathogenesis (4, 5) and as platforms to explore new therapies.

One of the first protocols to generate cardiomyocytes from hESCs was the co-culture of these cells on mouse visceral endoderm-like cells (END-2) (6). Although this method is effective for many human pluripotent stem cell lines (hPSCs), the yield of cardiomyocytes is low and is only effective with mechanically passaged hPSC lines on mouse embryonic fibroblasts (MEFs) (7). The adaptation of hESCs to single cell enzymatic passaging has provided alternative approaches to derive cardiomyocytes. One of these involves forming small aggregates of enzymatically adapted hPSCs in suspension by the centrifugation of these undifferentiated cells in low-attachment 96-well plates (8). This method, commonly referred to as either "forced aggregation" or "spin embryoid bodies," has significantly increased the yield of cardiomyocytes in culture as the initial number of hPSCs in each aggregate is controlled and identical across all wells. These protocols necessitate that the culture medium is serum-free and is usually a chemically defined medium that contains albumin, polyvinyl alcohol, and essential lipids (9). An animal component-free version of this basal medium containing only recombinant human proteins (APEL medium) is also commercially available. Alternatively, a micro-textured surface, also known as micro- or Aggrewells, has provided a scalable method for the initial formation of embryoid bodies of uniform size (10). Early protocols to generate cardiomyocytes using this technology required the use of serum, but the hPSCs can now be differentiated in serum-free medium with the addition of growth factors (11). Since 2012 significant progress has also been made with monolayer-based differentiation protocols leading to further improvements in efficiency and homogeneity of the cardiomyocytes generated, and this is rapidly becoming the preferred cardiac differentiation approach (12–14).

Common among all of these serum-free methods is the sequential mimicking in culture of key embryonic developmental signals to induce cardiac differentiation (7). Signals induced by nodal (using Activin A as a substitute), bone morphogenic proteins (BMPs), Wnts, and fibroblast growth factors (FGFs) are first required to generate cardiac mesoderm-like cells. Subsequent inhibition of the Wnt/β-catenin pathway, initially by Dickkopf-1 (Dkk-1), is then required for cardiac specification (15). Recently it has been shown that small molecules that lead to the activation and inhibition the Wnt/β-catenin pathway can replace some or all of the cytokines previously used (12, 13). However, the success of these small molecule-based methods is both highly concentration and time dependent, can vary between different cell lines, and is also sensitive to the method by which the undifferentiated cells are maintained.

In this chapter, we describe a monolayer-based protocol for the generation of cardiomyocytes from hPSCs that have been

maintained undifferentiated using a range of different approaches: either as single cell-passaged cultures on MEFs or in completely defined Essential 8 (E8) medium; or as mechanically passaged aggregates in mTeSR1 medium or on MEFs. We use BMP4 and Activin A together with CHIR99021, a glycogen synthase kinase (GSK-3) small molecule inhibitor that activates Wnt/β-catenin signaling, to induce cardiac mesoderm. This is followed by the temporal treatment with the tankyrase inhibitor XAV939, which inhibits Wnt/β-catenin signaling and leads to the efficient generation of cardiac progenitors. Approximately 1 week after initiating the differentiation, we observe spontaneously contracting cardiomyocytes. Combining both cytokines and small molecules in our differentiation protocol has proven to be very robust in our laboratory, producing high yields of cardiomyocytes from multiple hPSC lines (16, 17).

These cardiomyocytes can be used in many different assays including electrophysiology, optical mapping and force of contraction measurements for functional analysis, cell imaging and immunohistochemistry for structural and phenotypic analysis, as well as next-generation sequencing and mass spectrometry for global transcriptome and proteome analysis. We also describe here three established protocols to identify and quantify cardiomyocytes within the differentiated culture.

2 Materials

For culture recipes and steps, use sterile tissue culture-grade water. For other protocol steps, use deionized water or equivalent.
All cells are maintained and differentiated in a 37 °C humidified 5 % CO_2 incubator.
All differentiations are performed using 12- or 6-well tissue culture plates.

2.1 Cell Culture Medium and Reagents for Differentiation and Dissociation

- Phosphate buffered saline without $CaCl_2$ and $MgCl_2$ (PBS; Gibco).
- 0.5 mM EDTA (Invitrogen) in PBS.
- Dimethyl sulfoxide (DMSO; Sigma-Aldrich).
- 100 ng/mL human FGF-2 (Miltenyi Biotec) Stock Solution: reconstitute lyophilized powder according to the manufacturer's protocol. Keep cold at all times. Store aliquots at −80 °C for long-term storage for up to a year and keep thawed aliquots at 4 °C for up to 2 weeks.
- 25 ng/μL Recombinant human Activin A (R&D) Stock Solution: reconstitute lyophilized powder according to the manufacturer's protocol. Keep cold at all times. Store aliquots at

−80 °C for long-term storage for up to a year and keep thawed aliquots at 4 °C for up to 2 weeks.

- 25 ng/μL Recombinant human BMP4 (R&D) Stock Solution: reconstitute lyophilized powder according to the manufacturer's protocol. Keep cold at all times. Store aliquots at −80 °C for long-term storage for up to a year and keep thawed aliquots at 4 °C for up to 2 weeks.
- 4 mM CHIR99021 (Axon Medchem) Stock Solution: dissolve powder in DMSO. Store aliquots at −20 °C for long-term storage for up to a year and keep thawed aliquots at 4 °C for up to 3 weeks.
- 5 mM XAV939 (Tocris) Stock Solution: dissolve powder in DMSO. Store aliquots at −20 °C for long-term storage and keep thawed aliquots at 4 °C for up to 3 weeks.
- hESC medium: DMEM/F12 (Gibco) containing 20 % Knockout serum replacement (Gibco), 10 mM Minimal essential medium non-essential amino acids (Gibco), 2 mM L-glutamine (Gibco), 50 mM 2-mercaptoethanol (Gibco), 0.5 % Penicillin/streptomycin (25 units Penicillin G and 25 μg Streptomycin sulfate; Gibco), 10 ng/mL FGF-2. Filter sterilize and store at 4 °C for up to 2 weeks.
- mTeSR™1 medium (STEMCELL Technologies).
- Essential 8™ medium (Gibco).
- 1 mg/mL dispase (Gibco): dissolve powder as 5 mg/mL in DMEM/F12. Store aliquots at −20 °C for long-term storage for up to a year. Dilute thawed aliquots to 1 mg/mL in DMEM/F12 and keep at 4 °C for up to 2 weeks.
- TrypLE™ Select (1×) or TrypLE™ Select (10×) (Gibco): 10× solution can be further diluted to 2× or 5× concentration with PBS containing 1 mM EDTA.
- 10 % w/v bovine serum albumin (BSA, Bovostar; Bovogen Biologicals) Stock Solution (*see* **Note 1**): dissolve 4.5 g BSA in 35 mL of IMDM in a 50 mL conical tube at 37 °C and vortex to mix. Add IMDM up to 45 mL. Filter sterilize and store at 4 °C for up to 3 months.
- 150 mM α-Monothioglycerol (α-MTG; Sigma-Aldrich): add 13 μL α-Monothioglycerol per 1 mL IMDM. Store at 4 °C for up to 4 weeks.
- Differentiation medium: the basal medium consists of Iscove's Modified Dulbecco's Medium (L-glutamine, 25 mM HEPES, no phenol red (IMDM); Gibco) and F12 Nutrient Mixture (Ham) with GlutaMAX (Gibco) in a 1:1 ratio mixture. The medium is supplemented with 5 % (v/v) Protein-Free Hybridoma Medium (PFHM-II; Gibco), 0.25 % w/v BSA Stock Solution, 1× Chemically Defined Lipid Concentrate (100× stock; Gibco), 0.1× Insulin-Transferrin-Selenium-X

Supplement (100× stock; Gibco), 450 μM α-MTG, 0.05 mg/mL L-Ascorbic acid 2-phosphate (Sigma-Aldrich), 2 mM GlutaMAX supplement (100× stock, Gibco), 0.5 % Penicillin/streptomycin (25 units Penicillin G and 25 μg Streptomycin sulfate). Filter sterilize and store at 4 °C for up to 2 weeks or at −20 °C for up to 6 months. To set up new differentiations, use medium that is less than 1 week old.

- Matrigel™ Matrix Growth Factor Reduced (BD): dilute 0.5 mg Matrigel in 6 mL cold DMEM/F12. Use a cold pipet to transfer the solution to a 6- or 12-well plate (1 or 0.5 mL per well respectively). Leave at room temperature for at least 45 min to polymerize. Plates can be used immediately or stored at 4 °C for up to 2 weeks. Store Matrigel stock solution as 0.5 mg aliquots at −20 °C.
- Dilution medium: 10 % (v/v) fetal bovine serum (FBS; Gibco) in DMEM/F12.

2.2 Flow Cytometry

- Wash buffer: 2 % (v/v) FBS in PBS.
- Blocking solution: PBS containing (v/v) 2 % FBS, 2 % normal rabbit serum (Sigma-Aldrich), 1 % normal goat serum (DAKO). Solution can be stored at 4 °C for up to 3 months or at −20 °C indefinitely.
- Fixation medium and Permeabilization medium: FIX & PERM Cell Permeabilization Kit (Invitrogen).
- 4′,6-Diamidino-2-Phenylindole (DAPI; Invitrogen): 0.1 mg/mL in wash buffer.
- 1 mg/mL propidium iodide (PI; MP Biomedicals) in deionized water.
- Antibodies for cardiac Troponin T (TNNT2) detection are listed in Table 1 (*see* **Notes 2** and **3**).
- Antibodies for SIRPA and VCAM1 detection are listed in Table 2 (*see* **Notes 2** and **3**).

2.3 Immunofluorescence

- Fixation solution: 2 % paraformaldehyde in 0.2 M phosphate buffer (pH 7.4).
- Permeabilization solution: 0.1 % Triton X-100 (Sigma-Aldrich) in PBS.
- Blocking solution: 4 % (v/v) swine-serum (DAKO) in PBS (prepare fresh).
- Washing solution: 0.05 % (v/v) Tween20 (Merck) in PBS.
- Mowiol 4-88 (Calbiochem): dissolve according to the manufacturer's protocol.
- DAPI: 5 mg/mL stock solution in deionized water.
- Antibodies for immunofluorescence are listed in Table 3 (*see* **Note 4**).

Table 1
Antibodies for flow cytometric analysis for TNNT2

Primary and secondary antibody	Dilution	Supplier and catalogue nr.
Mouse-IgG1 isotype control	1:1,000	DAKO X0931
Mouse-anti-rabbit cardiac Troponin T, Isoform Ab-1, IgG1	1:1,000	Thermo Scientific MS-295-P1
Allophycocyanin (APC) conjugated donkey-anti-mouse IgG	1:100	Jackson ImmunoResearch 715-136-151

Table 2
Antibodies for flow cytometric analysis for SIRPA and VCAM1

Primary antibody	Dilution	Supplier and catalogue nr.
APC conjugated recombinant human IgG1 SIRPA (CD172a)	1:20	Miltenyi Biotec 130-099-785
APC conjugated mouse-anti-rat IgG1, isotype control	1:20	Miltenyi Biotec 130-095-902
PE conjugated mouse-anti-human IgG1, κ VCAM1 (CD106)	1:20	BD Bioscience 561679
PE conjugated mouse IgG1, isotype control	1:20	Miltenyi Biotec 130-092-212

Table 3
Antibodies for immunohistochemical analysis of cardiac sarcomeric proteins

Primary and secondary antibody	Dilution	Supplier and catalogue nr.
Mouse-anti-rabbit α-Actinin (sarcomeric) IgG1	1:800	Sigma Aldrich A7811
Rabbit-anti-human Troponin I IgG	1:500	Santa Cruz sc-15368
Cy3 conjugated goat-α-mouse IgG	1:250	Jackson ImmunoResearch 115-165-146
Alexa Fluor 488 conjugated donkey-α-rabbit IgG	1:100	Invitrogen A-21206

3 Methods

3.1 Seeding hPSCs for Differentiation

3.1.1 From Mechanically Passaged hPSC Cultures Maintained on MEFs

1. Maintain hPSCs as colonies on irradiated MEFs in hESC medium as described elsewhere (18).
2. 1–2 days before differentiation, cut each colony using a 26-G needle into ~15 pieces and dislodge from the dish. Transfer pieces in hESC medium onto a Matrigel-coated culture dish (~10 pieces per 4 cm^2) (Fig. 1a).
3. Refresh hESC medium daily (*see* **Note 5**).
4. When the culture is ~70 % confluent (*see* **Note 6**), proceed to the differentiation protocol (*see* Sect. 3.2).

3.1.2 From Enzymatically Passaged hPSC Cultures Maintained on MEFs

1. Maintain hPSCs on irradiated MEFs in hESC medium as described elsewhere (18).
2. The day before differentiation (d0 minus 1), the hPSC culture should be ~85 % confluent. Harvest the hPSCs by aspirating the hESC medium, rinsing once with PBS and adding sufficient 1× TrypLE Select to cover the surface area. Incubate for 5 min at 37 °C before adding 5× volume of hESC medium. To dissociate the hPSCs into single cells, gently pipette the cells up and down 3–5 times using a 5 mL Pasteur pipette.
3. Pellet the cells by centrifuging the tube for 3 min at 250 × *g*.
4. Aspirate the supernatant and resuspend the cells in hESC medium.
5. Plate cells on Matrigel-coated plates so that the culture is ~40 % confluent the next day (Fig. 1b) (*see* **Notes 6** and **7**).
6. Proceed to the differentiation protocol (*see* Sect. 3.2).

3.1.3 From hPSC Cultures Maintained in E8 Medium

1. Maintain hPSCs in E8 medium on tissue culture dishes pre-coated with vitronectin as described elsewhere (19).
2. 3–4 days before differentiation, the hPSC culture should be ~85 % confluent. Harvest the hPSCs by aspirating the E8 medium, rinsing once with PBS, and adding 0.5 mM EDTA to cover the surface area. Incubate for 5 min at 37 °C and aspirate EDTA when the colonies round up and holes start to appear.
3. Add sufficient E8 medium to the tissue culture dish to dissociate the hPSCs using a 5 mL Pasteur pipette. Gently pipette the cells up and down 3–5 times until they are small clusters.
4. Seed hPSCs in E8 medium on Matrigel-coated culture dishes at a density such that the culture will be 70–90 % confluent 72–96 h after passaging (Fig. 1c) (*see* **Notes 6** and **7**).

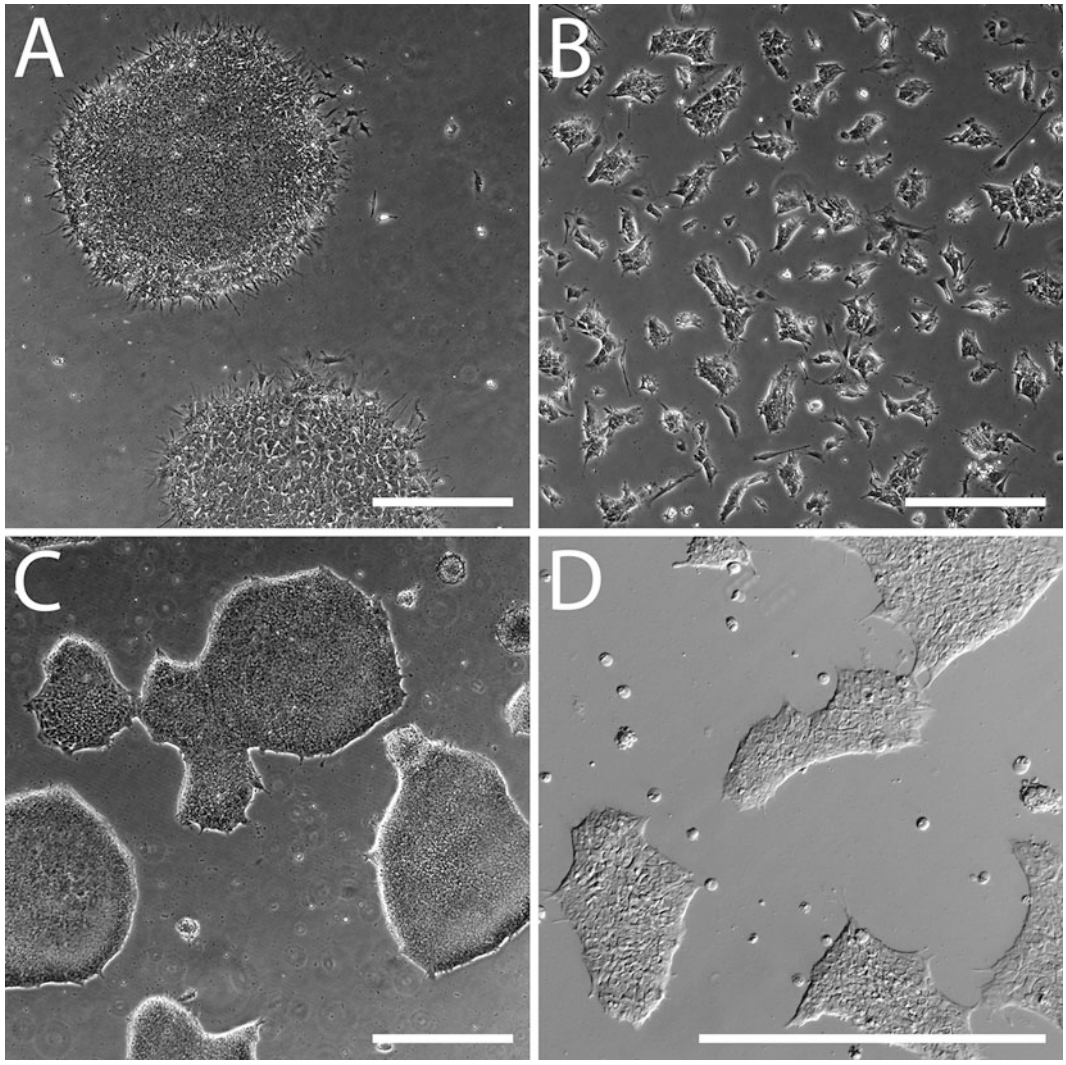

Fig. 1 Bright field images of hPSC morphology when seeded on Matrigel-coated dishes from (**a**) mechanically passaged hPSC cultures maintained on MEFs, (**b**) enzymatically passaged hPSC cultures maintained on MEFs, (**c**) hPSC cultures maintained in Essential 8 medium, (**d**) hPSC cultures maintained in mTeSR1. Scale bar: 500 μm

5. Refresh the E8 medium 48 h after passaging and repeat daily (*see* **Note 8**).
6. Proceed to the differentiation protocol (*see* Sect. 3.2).

3.1.4 From hPSC Cultures Maintained in mTeSR1

1. Maintain hPSCs in mTeSR1 on Matrigel-coated tissue culture dishes according to the manufacturer's protocol.
2. 3–4 days before differentiation, the hPSC culture should be ~80 % confluent. Cut each colony using a 26-G needle into four pieces.

3. Harvest the hPSCs by aspirating the mTeSR1 medium and adding dispase to cover the surface area. Incubate for 5 min at 37 °C and aspirate dispase when the colonies round up.
4. Rinse the well twice using 2× volume DMEM/F12 medium and aspirate. Add sufficient DMEM/F12 to dislodge the colony pieces from the dish. Transfer the pieces to a Matrigel-coated culture dish (~25–30 pieces per 4 cm^2) in mTeSR1 medium at a density such that the culture will be ~80 % confluent 72–96 h after passaging (Fig. 1d) (*see* **Note 6**).
5. Refresh medium daily with mTeSR1 until start of differentiation.
6. Proceed to the differentiation protocol (*see* Sect. 3.2).

3.2 Differentiating hPSCs to Cardiomyocytes

1. On day 0 of differentiation, aspirate hPSC maintenance medium and add differentiation medium supplemented with 20 ng/mL Activin A, 20 ng/mL BMP4, and 1.5 μM CHIR99021 (Fig. 2) (*see* **Note 9**). For a 12-well culture plate add 1.5 mL of differentiation medium per well, while for a 6-well culture plate add 3.5 mL of differentiation medium per well.
2. On day 3 of differentiation, the cells will have formed a monolayer covering the entire surface of the well (Fig. 2). Aspirate medium from each well and replace with an equal volume of differentiation medium supplemented with 5 μM XAV939.

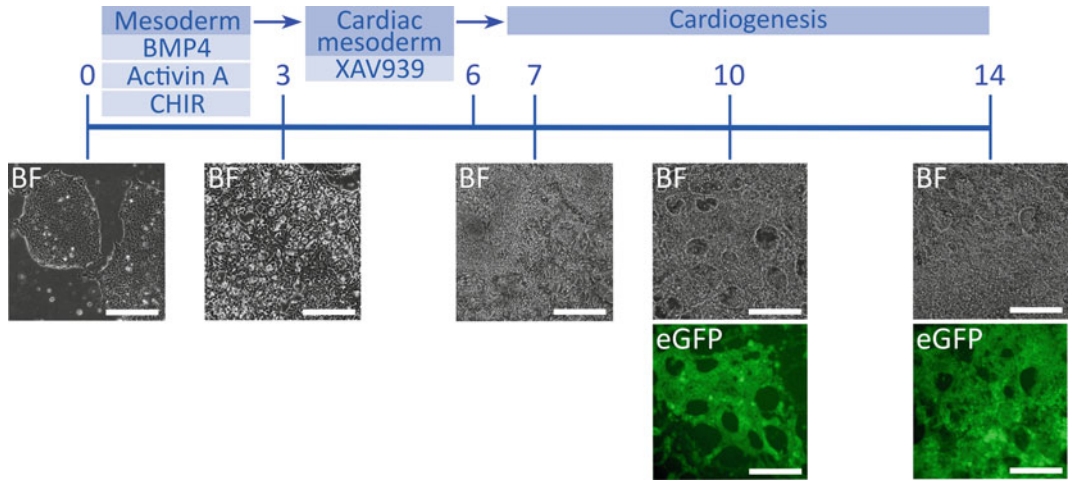

Fig. 2 Schematic representation of the protocol to differentiate hPSCs to cardiomyocytes. Bright field (BF) and green fluorescence (eGFP) images show the typical morphology of the differentiating cells at key stages during the differentiation. By day 7 of differentiation, web-like colonies begin to appear that subsequently start to contract by day 10 of differentiation (*upper panel* of images). If using an NKX2-5–eGFP reporter hESC or hiPSC line (8) (van den Berg, Davis, unpublished), these contracting cells will be GFP^+ (*lower panel* of images). Scale bar: 500 μm

3. On day 6 of differentiation, aspirate medium and replace with an equal volume of fresh differentiation medium (unsupplemented).
4. Refresh the differentiation medium every 3–4 days as described in step 3. Between days 7–9 of differentiation, spontaneously contracting areas within the well should be apparent (*see* **Notes 10** and **11**).
5. Wells containing spontaneously contracting areas can be maintained for at least 3 months. These regions can be dissociated (*see* Sect. 3.3), and the cardiomyocytes used for downstream assays, such as flow cytometry and immunohistochemistry.

3.3 Dissociating Cultures Containing hPSC-Derived Cardiomyocytes

1. Aspirate the medium and wash the cells with 1 mL PBS per well of a 12-well plate, or 3 mL PBS per well of a 6-well plate. Aspirate the PBS solution.
2. Add 0.5 mL TrypLE Select per well of a 12-well plate, or 1 mL TrypLE Select per well of a 6-well plate. Incubate the culture for 5–15 min at 37 °C (*see* **Note 12**).
3. To detach the cells, gently tap the plate with your palm. If gaps are visible in the sheet of cells or it detaches from the well, use a 1 mL pipette to carefully pipet the suspension up and down 3–5 times. If the cells have not started to detach, incubate the plate at 37 °C for a further 5 min and reassess. Continue to do this until the cells detach.
4. Transfer the single cell suspension to a 15 mL conical tube containing 5 mL of dilution medium.
5. Centrifuge the cells for 3 min at 450 × *g*, 4 °C.
6. Aspirate the supernatant and resuspend the cells in cold wash buffer for flow cytometric evaluation (*see* Sect. 3.4 or 3.5) or differentiation medium for immunohistochemical analysis (*see* Sect. 3.6).

3.4 Flow Cytometric Analysis for Cardiac TNNT2 (Intracellular Cardiac Marker)

1. Filter the cell suspension using a 5 mL tube with a 35 μm cell strainer cap.
2. Pellet the cells by centrifuging the tube for 3 min at 450 × *g*, 4 °C. Aspirate the wash buffer.
3. Resuspend the cells in 100 μL Fixation Medium and incubate for 15 min at room temperature according to the manufacturer's protocol.
4. Add 3 mL cold wash buffer to the cells and centrifuge the tube for 3 min at 450 × *g*, 4 °C. Aspirate the supernatant.
5. Resuspend the cell pellet in 300 μL Permeabilization Medium by vortexing the tube. Evenly divide the cell suspension across three tubes (100 μL per tube).

Table 4
Control and experimental tubes for flow cytometric analysis of TNNT2 expression

	First incubation	Second incubation
Tube 1	Unstained control	–
Tube 2	IgG1 isotype control	APC conjugated antibody
Tube 3	Cardiac Troponin T	APC conjugated antibody

6. Dilute the primary antibodies 1:10 in Permeabilization Medium and add 1 μL of the prediluted primary antibodies to the tubes as indicated in Table 4. Tubes 1 and 2 are control samples.
7. Briefly vortex the tubes and incubate for 30 min at room temperature.
8. Wash the cells by adding 3 mL of cold wash buffer to each tube. Centrifuge the tubes for 3 min at 450 × *g*, 4 °C. Aspirate the supernatant. Repeat this step.
9. Add 300 μL wash buffer to tube 1 and keep cold until flow cytometry measurement.
10. Add 2 μL of the secondary antibody to 200 μL Permeabilization Medium and add 100 μL of the diluted secondary antibody to tubes 2 and 3.
11. Briefly vortex the tubes to resuspend the cell pellet and incubate for 20 min in the dark at room temperature.
12. Wash the cells by adding 3 mL of cold wash buffer to each tube. Centrifuge the tubes for 3 min at 450 × *g*, 4 °C. Aspirate the supernatant. Repeat this step.
13. Resuspend the cell pellet in 300 μL wash buffer and keep tubes cold.
14. Set up the flow cytometer using tubes 1 and 2 as instrument and gating controls.
15. Perform flow cytometric evaluation of TNNT2 expression (Fig. 3a).

3.5 Flow Cytometric Analysis for SIRPA and VCAM1 (Cell Surface Markers)

1. Filter the cell suspension using a 5 mL tube with a 35 μm cell strainer cap.
2. Pellet the cells by centrifuging the tube for 3 min at 450 × *g*, 4 °C. Aspirate the wash buffer.
3. Resuspend the cell pellet in 500 μL blocking buffer by vortexing. Evenly divide the cell suspension across five tubes (100 μL per tube) labeled according to Table 5. Tubes 1–4 are control samples and are used to set up the flow cytometer.

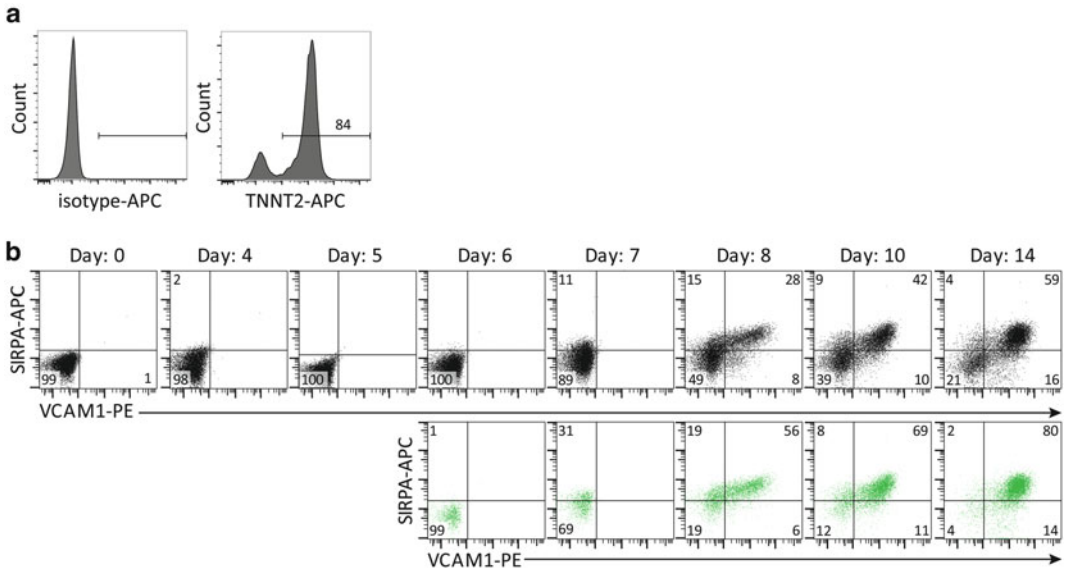

Fig. 3 Characterization and quantification of hPSC-derived cardiomyocytes by flow cytometry. (**a**) Representative histograms of the proportion of TNNT2$^+$ cells at day 21 of differentiation. The *graph* on the *left* shows the isotype control and was used to establish the gating strategy. The percentage of TNNT2$^+$ cells is indicated in the *graph* on the *right*. (**b**) Time course of SIRPA and VCAM1 expression between days 0 and 14 of differentiation for the whole cell population (*upper panel*) or within the GFP$^+$ (NKX2.5$^+$) population (*lower panel*) of an NKX2-5–eGFP reporter hPSC line. The proportion of cells in each quadrant is indicated

Table 5
Control and experimental tubes for flow cytometric analysis of SIRPA and VCAM1 expression

	First incubation	Second incubation
Tube 1	Unstained control	–
Tube 2	SIRPA (APC conjugated)	–
Tube 3	VCAM1 (PE conjugated)	–
Tube 4	Isotype control (APC conjugated)	Isotype control (PE conjugated)
Tube 5	SIRPA (APC conjugated)	VCAM (PE conjugated)

4. Add the appropriate dilution of each antibody (Table 2) to the tubes.
5. Briefly vortex the tubes and incubate for 20 min in the dark on ice.
6. Wash the cells by adding 3 mL of cold wash buffer to each tube. Centrifuge the tubes for 3 min at 450 × *g*, 4 °C. Aspirate the supernatant. Repeat this step.

7. Add 300 μL wash buffer to tube 1–3 and keep cold until flow cytometry measurement.
8. Resuspend the cell pellet in tubes 4 and 5 in 100 μL blocking buffer and add 5 μL of the appropriate antibody according to Table 2.
9. Briefly vortex the tubes and incubate for 20 min in the dark on ice.
10. Wash the cells by adding 3 mL of cold wash buffer to each tube. Centrifuge the tubes for 3 min at 450 × *g*, 4 °C. Aspirate the supernatant. Repeat this step.
11. Resuspend the cell pellet of tube 4 and 5 in 300 μL wash buffer and keep tubes on ice.
12. Perform flow cytometry measurement. Set up the flow cytometer using tubes 1–4 as instrument, gating, and compensation controls (Fig. 3b) (*see* **Note 13**).

3.6 Immunohistochemical Analysis of Sarcomeres in hPSC-Derived Cardiomyocytes

1. Place a sterile glass coverslip (15 mm) in each well of a 12-well plate. Coat the glass coverslips with Matrigel.
2. Count the dissociated cells using a hemocytometer. Make a cell suspension of 1×10^6 cells/mL.
3. Remove the Matrigel solution and plate $5–7.5 \times 10^5$ cells per well (50–75 μL) in a total of 1.5 mL of differentiation medium (*see* **Note 7**).
4. Five days after seeding the cells, replace the medium with 1.5 mL of fresh differentiation medium.
5. Approximately 10–15 days after seeding, fix the cells (*see* **Note 14**). Aspirate the differentiation medium from each well and wash the cells once with 1 mL PBS. Aspirate the PBS and add 1 mL fixation solution per well in a fume hood. Incubate the cells for 30 min at room temperature.
6. Remove fixation solution and wash the cells three times with 1 mL PBS. The fixed cells can be immunostained immediately for sarcomeric markers or stored at 4 °C for 2–3 weeks in 1.5 mL PBS per well.
7. Rinse the cells briefly with PBS.
8. Add 1 mL permeabilization solution per well for 8 min at room temperature to permeabilize the cells.
9. Aspirate the permeabilization solution and wash the coverslips three times with PBS.
10. Remove coverslips and place on parafilm strips in a sealed humidified container.
11. Preincubate the cells with 100 μL blocking solution per coverslip for 1 h at room temperature.

12. Prepare 100 μL blocking solution per coverslip containing both primary antibodies according to Table 3.
13. Remove the blocking solution from each coverslip and add 100 μL of the primary antibody solution to each coverslip. Incubate overnight at 4 °C.
14. Wash the cells with 200 μL washing solution per coverslip for 10 min. Remove the washing solution. Repeat twice.
15. Prepare 100 μL blocking solution per coverslip containing both secondary antibodies according to Table 3.
16. Add 100 μL of the diluted secondary antibody solution to each coverslip. Incubate at room temperature for 1 h.
17. Wash the cells with 200 μL washing solution per coverslip for 20 min. Remove the washing solution. Repeat twice.
18. Incubate the cells for 5 min at room temperature with 100 μL DAPI diluted 1:1,000 in blocking solution to stain the nuclei.
19. Rinse the cells briefly in H_2O.
20. Seal coverslips to glass microscope slides using a small drop (~10 μL) of Mowiol. Leave slides to dry overnight in the dark.
21. Examine the sarcomeric organization of hPSC-derived cardiomyocytes using an epifluorescence or confocal microscope (Fig. 4).

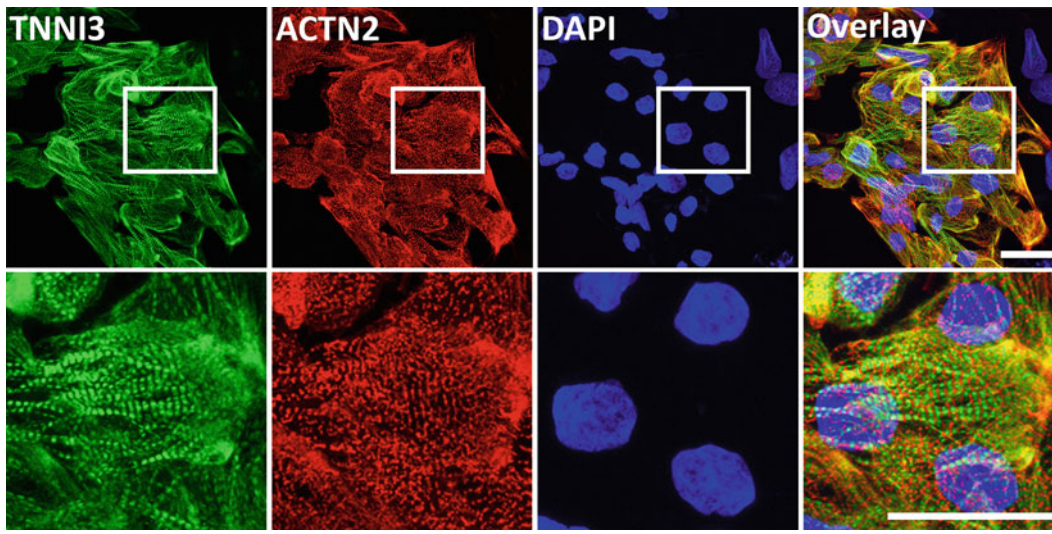

Fig. 4 Identification of hPSC-derived cardiomyocytes by immunohistochemistry. Confocal immunofluorescence images of Troponin I (TNNI3; *green*) and α-actinin (ACTN2; *red*) to visualize the sarcomeres in the cardiomyocytes. All nuclei were stained with DAPI (*blue*). The images in the *bottom panel* are magnifications of the area framed in the *upper images*. Scale bar: 25 μm

4 Notes

1. The quality of the BSA can vary between different batches and between companies (9). Several sources of BSA should be tested initially. To reduce the cytotoxicity of the BSA, a deionization step using Resin Beads (Bio-Rad, AG 501-X8(D) Resin #142-6425, 20–50 dry mesh size) can be performed if necessary (9).
2. We prefer to use primary antibodies that are directly conjugated to a fluorescent label. However the primary antibodies can also be detected using labeled conjugated secondary antibodies. If the expression of an epitope is low, this approach can improve the signal to background intensity.
3. We describe two protocols for flow cytometric analysis for either intracellular or cell-surface proteins expressed in hPSC-derived cardiomyocytes. TNNT2 expression is frequently examined by intracellular staining to assess the yield of hPSC-derived cardiomyocytes but cannot purify the cardiomyocytes for further culturing. Furthermore, while TNNT2 and NKX2-5 (mirrored by GFP (8)) are usually co-expressed in hPSC-derived cardiomyocytes, we have observed in differentiations older than 21 days a population of $TNNT2^{+}$ cells that is $NKX2\text{-}5^{-}$ (i.e., GFP^{-}). The use of a second cardiomyocyte marker, such as α-actinin, also could be considered. Alternatively, the combined labeling for the cell surface proteins SIRPA and VCAM1 enriches for a population of viable cardiomyocytes that can be isolated by flow cytometry and subsequently cultured further (8, 20).
4. Dissociated single cardiomyocytes can be stained for two sarcomeric markers. Troponin I combined with α-actinin displays the characteristic overlap of sarcomeres.
5. Cultures of undifferentiated hPSCs cannot be maintained in hESC medium without MEFs for more than 48 h. If the cultures do not reach 70 % confluence within this timeframe, we suggest either increasing the initial cell seeding density or seeding the hPSCs in mTeSR1 or E8 medium. The cells can be maintained in these defined media for up to 5 days before starting the differentiation procedure.
6. The density of the cells at day 0 of the differentiation is a key factor in determining the efficiency of the differentiation and in obtaining a high yield of cardiomyocytes. The culture should be ~50–80 % confluent at the start of the differentiation but should be independently assessed for each hPSC line. From enzymatic passaged hPSCs maintained on MEFs, we typically seed 2.5×10^4 hPSCs/cm^2, while for hPSCs maintained in E8 medium a confluent culture would be passaged between 1:6

and 1:10. There is variability in the growth rate between different hPSC lines and the optimal cell seeding density will need to be determined for each hPSC line individually. We recommend initially testing three different seeding densities.

7. Inhibiting Rho-associated protein kinase (ROCK) using either 10 μM Fasudil (HA1077) or 10 μM Y-27632 when seeding hPSCs or hPSC-derived cardiomyocytes can reduce dissociation-induced apoptosis (21, 22). Refresh medium 24 h after seeding to remove the inhibitor.
8. For some hPSC lines we have observed significant cell death and detachment once the differentiation procedure commences, especially with hPSCs maintained in E8 medium. Treating the seeded cells with E8 medium containing 1–2 % DMSO for 24–30 h before the start of the differentiation can improve cell survival and cardiac differentiation efficiency (23).
9. The bioactivity of cytokines can vary between different batches and suppliers. The optimal concentration of BMP4 and Activin A for generating cardiomyocytes can be determined by cross-titrating these growth factors (8, 24).
10. If the differentiated hPSCs form a sheet of cells that are spontaneously contracting across the entire well, the culture can be overlaid with Matrigel to reduce the likelihood of the cells detaching from the plate. This additional layer of Matrigel can be applied at any time after day 3 of differentiation, including when the cells are treated with XAV939. Add 1 mg of thawed Matrigel to 12 mL of cold differentiation medium. Immediately aspirate the medium from the well and add an equal volume of the Matrigel-containing differentiation medium to each well.
11. Genetically engineered reporter hESC and hiPSC lines in which the expression of a fluorescent protein, such as eGFP or mCherry, is under the control of a cardiac-specific promoter are useful tools that are available to rapidly evaluate the progress and efficiency of the cardiac differentiation in real time (8, 17, 25–27) (van den Berg, Davis, unpublished). Furthermore, these reporters can facilitate the isolation of relatively pure cardiomyocyte populations for further culturing.
12. The concentration of TrypLE Select and incubation time needed to dissociate the differentiated hPSCs are dependent on the age of the differentiation. We generally use 1× TrypLE Select (incubation time ~5 min) to dissociate cultures that have been differentiated for less than 8 days; 2× TrypLE Select (incubation time ~10 min) for differentiations between 9 and 12 days; 5× TrypLE Select (incubation time ~10 min) for differentiations between 13 and 18 days; and 10× TrypLE

Select (incubation time minimum 10 min) for cultures that have been differentiated for more than 19 days.

13. When performing flow cytometric analysis on live cells, a viability marker such as DAPI (0.1 μg/mL) or PI (1 μg/mL) can be used to exclude dead cells.
14. When seeded on coverslips the differentiated hPSCs should not become >70 % confluent due to the proliferation of noncardiomyocytes but should be fixed when the cardiomyocytes are still present as individual or small clusters of cells.

Acknowledgements

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Generation of Cardiomyocytes from Pluripotent Stem Cells

Hiroko Nakahama and Elisa Di Pasquale

Abstract

The advent of pluripotent stem cells (PSCs) enabled a multitude of studies for modeling the development of diseases and testing pharmaceutical therapeutic potential in vitro. These PSCs have been differentiated to multiple cell types to demonstrate its pluripotent potential, including cardiomyocytes (CMs). However, the efficiency and efficacy of differentiation vary greatly between different cell lines and methods. Here, we describe two different methods for acquiring CMs from human pluripotent lines. One method involves the generation of embryoid bodies, which emulates the natural developmental process, while the other method chemically activates the canonical Wnt signaling pathway to induce a monolayer of cardiac differentiation.

Keywords: Pluripotent stem cells, Human cardiomyocytes, Cardiac differentiation, EB method, Monolayer method

1 Introduction

Pluripotent stem cells (PSCs) are cells that are derived from the embryonic inner cell mass or cells that are induced into a pluripotent state by altering the pluripotent epigenetic landscape. These cells have been invaluable in investigating various diseases, especially in studies utilizing cells derived from non-regenerative tissues such as the heart (1). Especially in utilizing induced PSCs, patient-specific CMs can now be generated to model various types of cardiovascular diseases such as long QT syndrome (2–4), catecholaminergic polymorphic ventricular tachycardia (5–7), Brugada syndrome (8), and also cardiac-specific phenotypes of multi-organ system disorders such as Leopard syndrome (9) and Timothy syndrome (10). In investigating the development of cardiovascular diseases, deriving CMs remains the crucial bottleneck of the process. Two prominent methods of differentiation, the EB formation method and the monolayer method, have been established and enhanced to optimize CM generation.

The first established cardiac differentiation protocol employed the formation of cell aggregates called embryoid bodies (EBs). This method, utilizing fetal calf serum-based medium, has effectively differentiated various cell lines into CMs. Variations on the EB protocol have been established to improve the efficiency of cardiac

induction (11–16). Co-culture of visceral endoderm-like cells (END2) (12) and the addition of 5-aza-cytidine (13) had been shown to slightly improve differentiation. Furthermore, temporal addition of a combination of various growth factors such as bFGF, TGF-β, VEGF, BMP4, activin A, DKK-1, as well as canonical Wnt signaling proteins (14–16) has been performed to improve differentiation. During differentiation, sequential expression of mesodermal, cardiac progenitor, and cardiac-specific markers can be detected (17), allowing for developmental stagewise studies of cardiogenesis. However, the use of animal-based reagents such as fetal calf serum limits the use of the EB method due to the batch-to-batch variability of the serum and the inability to translate these cells into therapeutic use.

Other cardiac differentiation methods that generate highly pure populations of differentiated CMs via a monolayer of cells have been recently described (15, 18, 19). Similar to the EBs method, cardiac differentiation in a monolayer can also be induced by the addition of defined growth factors such as activin A in the early stages of differentiation and subsequent addition of BMP4 in a medium with RPMI containing B27 supplement (18) and in the presence of bFGF in an extracellular matrix "Matrigel sandwich" (19). Upregulating canonical Wnt signaling in the early stages of differentiation and its subsequent inhibition after several days has also been shown to enhance cardiogenesis (15). Although these methods are strictly dependent on the correct timing and concentration of growth factors that regulate cardiogenesis, this monolayer method allows us to reach very high yield of pure CMs and restricts the use of animal-derived serums, thus making them a more ideal method for potential translational studies.

Here, we present two efficient differentiation protocols: the first, a simplified EB method and, the second, a modified monolayer differentiation method. The EBs differentiation utilizes a medium containing fetal calf serum with the EBs in suspension for the first 7 days of differentiation and subsequent attachment and further differentiation on gelatin-coated plates. The monolayer differentiation method utilizes CHIR-99021 to inhibit glycogen synthase kinase 3 (GSK3) to activate the canonical Wnt signaling pathway and subsequently inhibiting the pathway 3 days later with the addition of IWR-1 (15). The former approach provides a quick and simple method for deriving CMs, whereas the latter approach generates up to 95 % of highly purified population of CMs with a completely xeno-free medium.

2 Materials

Prepare all solutions using cell culture-certified reagents and filter sterilize with 0.2 μm filters. Aliquot and store all medium and

reagents in appropriate temperatures as indicated. Prepare stock solutions of reagents first before preparing the medium or beginning experiments.

2.1 Embryoid Bodies

2.1.1 Stock Solution

1. Fetal bovine serum (FBS) South American origin (Life Technologies 10270-106): Heat inactivate FBS in a 56 °C water bath for 30 min before preparing aliquots. Store aliquots of 50 mL in −20 °C.
2. Ascorbic acid (Sigma A4544-25G) stock solutions of 10 mg/mL: Weigh 200 mg of ascorbic acid and transfer to a 50 mL conical tube. Add 20 mL of distilled water and filter sterilize. Store aliquots of 200 μL in −20 °C or keep in 4 °C for up to 2 weeks.
3. Dispase II (Roche 04942078001) stock solutions of 10 mg/mL: Dissolve 1 g of Dispase II in 100 mL of DMEM:F12 (1:1) (Life Technologies 11330-032), 5 mL at a time so as not to leave any powder in the bottle. Add solution to Stericup (Millipore or other similar filter sterilizing apparatus) and filter sterilize. Prepare aliquots of 10 mL in 15 mL conical tube and store in −20 °C.

2.1.2 Reagents and Other Materials

1. 250 mL Stericup (Millipore) or other 0.2 μm filter apparatus.
2. Embryoid body medium 20 (EBM20): DMEM:F12 (1:1), 20 % FBS South American origin, 1× GlutaMAX (Life Technologies 35050-038), 1× penicillin-streptomycin (Life Technologies 15140-122), 1× MEM nonessential amino acid (Life Technologies 11140-135), 50 μM β-mercaptoethanol (Life Technologies 31350-010), and 50 μg/mL ascorbic acid. In a 250 mL Stericup, add 192.5 mL of DMEM:F12 (1:1), 50 mL of FBS, 2.5 mL of GlutaMAX, 2.5 mL of penicillin-streptomycin, 2.5 mL of nonessential amino acid, and 250 μL of β-mercaptoethanol to make a total of 250 mL of medium. Filter and store in 4 °C. Before use, aliquot 50 mL of medium in a 50 mL conical tube and add 100 μL of the 10 mg/mL stock solution of ascorbic acid (this medium is denoted as EBM20 + AA).
3. Embryoid body medium 2 (EBM2): Follow the same instructions as EBM20 except replace 20 % FBS with 2 % FBS (add 5 mL of FBS instead of 50 mL). Also replace 192.5 mL of DMEM:F12 (1:1) with 237.5 mL of DMEM:F12 (1:1). Before use, aliquot 50 mL of medium in a 50 mL conical tube and add 100 μL of the 10 mg/mL stock solution of ascorbic acid (this medium is denoted as EBM2 + AA).
4. Dispase II 1 mg/mL: Add 5 mL of 10 mg/mL stock solution of Dispase II to 45 mL of PBS 1× in a 50 mL conical tube. Filter sterilize and store in 4 °C.

5. Cell scraper (Corning Incorporated 3008).
6. 6-well ultralow-attachment plate (Corning Incorporated 3471).
7. Gelatin 0.1 % in water (STEMCELL Technologies 07903).
8. 35 mm tissue culture-treated plates (Becton Dickinson 353001).

2.2 Wnt Pathway Monolayer Method

2.2.1 Stock Solution

1. BD Matrigel basement membrane matrix growth factor reduced (GFR) (BD Biosciences 354230): Thaw one bottle on ice overnight in 4 °C. Cool cryovials on ice and aliquot 500 μL of GFR Matrigel into each cryovial on ice. Store aliquots in −20 °C.
2. Accutase (Life Technologies A11105-01): Thaw a bottle of Accutase at room temperature and prepare aliquots of 10 mL in 15 mL conical tubes and store in −20 °C or in 4 °C for up to 2 weeks.
3. Y-27632 Rock inhibitor (Sigma-Aldrich Y0503-1MG) 5 mM stock solutions: Dissolve 1 mg of Y-27632 Rock inhibitor in 624.5 μL of water. Store aliquots of 50 μL in −20 °C or in 4 °C for up to 2 weeks.
4. CHIR-99021 (Selleck Chemicals S1263-5MG) 36 mM stock solutions: Dissolve 5 mg of CHIR-99021 in 1 mL of DMSO. Store aliquots of 20 μL in −80 °C.
5. IWR-1 (Sigma-Aldrich I0161-5MG) 5 mM stock solutions: Dissolve 5 mg of IWR-1 in 2.44 mL of DMSO. Store aliquots of 50 μL in −20 °C.

2.2.2 Reagents and Other Materials

1. 12-well tissue culture-treated plates (Becton Dickinson 353043).
2. GFR Matrigel: Dilute 1:30 in DMEM:F12 (1:1) by adding an aliquot of 500 μL to 15 mL of DMEM:F12 (1:1) in a 50 mL conical tube. Add 500 μL of solution to each well of a 12-well plate. Approximately 2.5 12-well plates can be prepared from one aliquot of Matrigel.
3. Essential 8 growth medium (Life Technologies A1517001).
4. Accutase: 1 mL per 35 mm plate.
5. Y-27632 Rock inhibitor: Add 10 μM from a stock solution of 5 mM into Essential 8 growth medium and filter sterilize. Prepare fresh every time before each treatment.
6. RPMI 1640 without insulin: RPMI 1640 (Life Technologies 21875-091), 50× B27 without insulin (Life technologies A18956-01), and 1× GlutaMAX. Add 212.5 mL of RPMI 1640, 5 mL of B27 without insulin, and 2.5 mL of GlutaMAX to a 250 mL Stericup and filter sterilize.

7. RPMI 1640 with insulin: Same as RPMI 1640 without insulin except replace 50× B27 without insulin with 50× B27 with insulin (Life Technologies 17504-044).
8. CHIR-99021: Add 8–12 μM from a stock solution of 36 mM into RPMI 1640 without insulin and filter sterilize (*see* **Note 1**). Prepare the differentiation solution containing the CHIR-99021 fresh before each treatment and filter sterilize.
9. IWR-1: Add 5 μM from a stock solution of 5 mM into RPMI 1640 without insulin and filter sterilize. Prepare fresh every time before each treatment.

3 Methods

Pre-warm all medium and reagents to room temperature before use.

3.1 Embryoid Body Method

A schematic representation of the protocol's steps is provided in Fig. 1.

1. Aspirate the medium from one 35 mm plate containing cells that have reached confluence (*see* **Note 2**).
2. Add 1 mL of Dispase and incubate for 2–5 min on 37 °C heated surface under the culture hood. Once the borders start to appear white under the stereomicroscope, gently aspirate out the Dispase (*see* **Note 3**).
3. Wash the plate once with 1 mL of DMEM:F12 (1:1) and discard. Add 1 mL of DMEM:F12 (1:1) and use a cell scraper to gently detach all of the cell colonies from the plate (*see* **Note 4**).

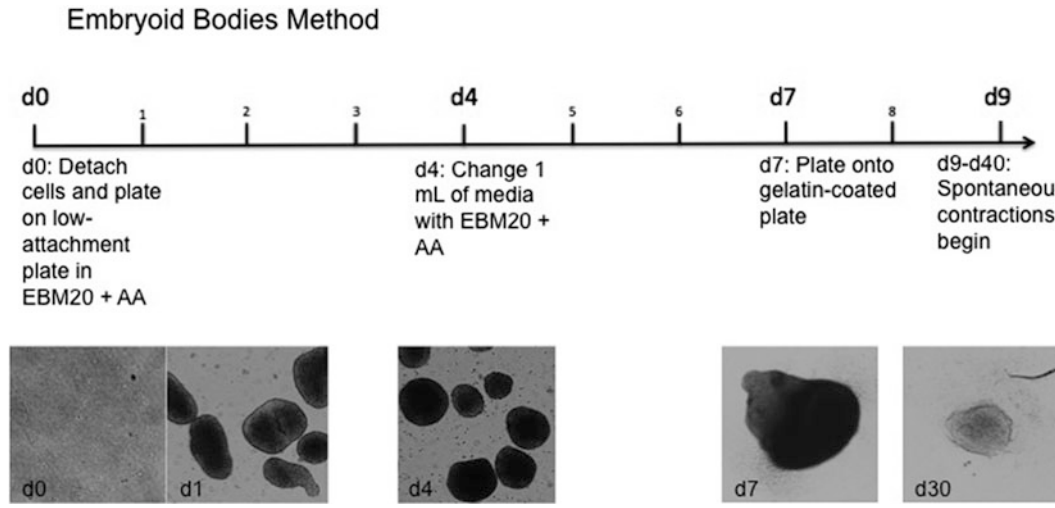

Fig. 1 Timeline of embryoid body method from day 0 to day 9 with corresponding light microscope images taken at 4× magnification

4. Use a 2 mL pipette to transfer cells into a 15 mL conical. Wash plate once with DMEM:F12 (1:1) and add to 15 mL conical. Centrifuge at 200 × *g* for 4 min.
5. Remove supernatant and resuspend pellet in 1 mL of EBM20 + AA. Use a 2 mL pipette and pipette up and down once or twice under the stereomicroscope, checking the size of the colonies (*see* **Note 5**). Plate cells at a 1:1 ratio (i.e., one 35 mm plate to 1 well of a 6-well plate) into the 6-well ultralow-attachment plate.
6. Wash 15 mL conical once with 1 mL of EBM20 + AA and add to well (*see* **Note 6**).
7. On day 4, remove 1 mL of medium and replace with 1 mL of fresh EBM20 + AA (*see* **Note 7**).
8. On day 7, coat 35 mm plates with 1 mL of 0.1 % gelatin and incubate in 37 °C incubator for 30 min. Remove gelatin and transfer 20–30 EBs into each gelatin-coated plate. Add 2 mL of EB20 + AA to each plate. If there are extra plates, remove the gelatin from the plates and they can be kept under the cell culture hood for up to a week.
9. Change the medium twice a week with 2 mL of EBM20 + AA and check for beating areas (*see* **Note 8**).
10. Once beating cardiomyocytes form, change the medium to EBM2 + AA and hereafter only use EBM2 + AA to change the medium.

3.2 Monolayer Method

Cells used for this protocol should be passaged with EDTA for at least three passages before starting experiment. Warm reagents to room temperature before use. Prepare GFR Matrigel-coated plates at least 1 day before plating. Plates can be kept in 4 °C for up to a week.

1. Thaw one aliquot of GFR Matrigel on ice. Once thawed, dilute the aliquot at a ratio of 1:30 in DMEM:F12 (1:1) by adding one 500 μL aliquot to 14.5 mL of DMEM: F12 in a 50 mL conical tube. Coat 12-well plates with 500 μL of Matrigel/DMEM mixture on ice. Cover plates with aluminum foil and store on ice in 4 °C overnight. Remove the ice the next day and place plates in 37 °C for at least 30 min before use.
2. *Day −4*:
   (a) Calculate the total amount of E8 medium that you will need and add 10 μM of Y-27632 rock inhibitor (Rocki) (*see* **Note 9**). Filter the medium. This medium will be denoted as E8 + Rocki.
   (b) Remove the old medium and add 1 mL of E8 + Rocki per plate and place plates back in incubator for 1 h to pretreat the cells with Rocki.

(c) After pretreatment, remove the medium and rinse once with 1 mL of PBS 1×. Add 1 mL of pre-warmed Accutase per plate and incubate for 2–5 min on 37 °C heated surface.

(d) Add 1 mL of E8 medium per well and pool all cells in a 15 mL conical tube. Centrifuge at 200 × *g* for 4 min at room temperature. Aspirate the supernatant and resuspend the cells in 1 mL of E8 + Rocki.

(e) Count cells. Seed 100,000 cells per well of Matrigel-coated 12-well plate. Bring the final volume to 1 mL per well with E8 + Rocki (*see* **Note 10**).

(f) Disperse the cells evenly by shaking the plate in quick, short side-to-side movements and place in 37 °C incubator (*see* **Note 11**).

3. Change the medium with 2 mL of E8 medium every day for the next 3 days.
4. *Day 0*: Aspirate the old medium. Add 2 mL of room temperature RPMI/B27 without insulin and add a final concentration of 8–12 μM of CHIR-99021 and filter sterilize (*see* **Note 12**). Record the time of treatment.
5. *Day 1*: After 24 h, aspirate the medium with p1000 tip and gently add fresh RPMI/B27 without insulin (*see* **Note 13**).
6. *Day 3*:

(a) Prepare 1.5 mL per well of fresh RPMI/B27 without insulin containing IWR-1 at a final concentration of 5 μM (calculated for a total of 3 mL of medium per well) and filter sterilize. Collect and add 1.5 mL of the old medium from each well into the 15 mL conical containing RPMI/B27 without insulin and IWR-1.

(b) Gently rock the plate back and forth to collect the remaining debris and aspirate out the rest of the medium.

(c) Add 3 mL of the combined medium containing IWR-1 to each well.

7. *Day 5*: Aspirate the old medium and gently add 2 mL of room temperature RPMI/B27 without insulin (*see* **Note 14**).
8. *Day 7*: Aspirate the old medium and gently add 2 mL of room temperature RPMI/B27 without insulin (*see* **Note 15**).
9. *Day 10*: Aspirate the old medium and gently add 2 mL of room temperature RPMI/B27 *with insulin*.
10. Continue to change the medium every 2–3 days with 2 mL of RPMI/B27 *with insulin*.

4 Notes

1. The optimal concentration of CHIR-99021 may vary between 8 and 12 μM to compensate for the sensitivity of each cell line. An initial test of treating cells at various concentrations in this range is suggested.
2. Cells should be passaged at around 60–75 % confluence without the colony of cells touching each other.
3. If the Dispase is left in the cells for too long, the cells will detach and the viability of the cells will decrease. On the other hand, if the incubation with the Dispase is too short, the cells will undergo too much mechanical damage when the cell scraper is used in step 3.
4. Use the cell scraper by applying a small downward pressure such that the cells can be removed as a whole colony.
5. Avoid over-pipetting as small colonies tend to disintegrate in the low-attachment well.
6. If the cells look like they are disintegrating the next day, add 500 μL of E8 per well to slowly adapt the cells to the EBM20 + AA differentiation medium. The next day, aspirate 1 mL of medium and replace with 1 mL of fresh EBM20 + AA medium.
7. To avoid aspirating out the EBs, swirl the plate in a slow, circular motion to collect the cells to the middle of the well. Use a p1000 pipette to aspirate the medium from the edge of the well where there are no cells.
8. Once a beating area is detected, mark the cover of the plate to locate the beating area under the stereomicroscope. Beating recedes soon after medium change but will resume beating after around 1 h. Beating areas may be observed as early as 2 days after plating and up to 1 month after plating.
9. For one 35 mm plate, you need 1 mL for pretreatment, 1 mL to resuspend pellet, and 1 mL per well of a 12-well plate. Calculate and prepare the appropriate amount of medium containing Rocki before starting.
10. Low seeding density will impede growth. If the cells do not attach and grow after the first day, consider seeding at a higher density at around 150,000–200,000 cells per well.
11. Single cells attach rapidly. If the cells are not dispersed evenly quickly after being plated, the uneven density can alter the growth and subsequent differentiation.
12. The confluence of the cells at the time of treatment should be at 95–100 %. The optimal concentration of CHIR-99021 may vary between cell lines based on their sensitivity to the treatment. Consider testing the range of CHIR-99021 from **Note 1** concentrations to the cell line before beginning experiment.

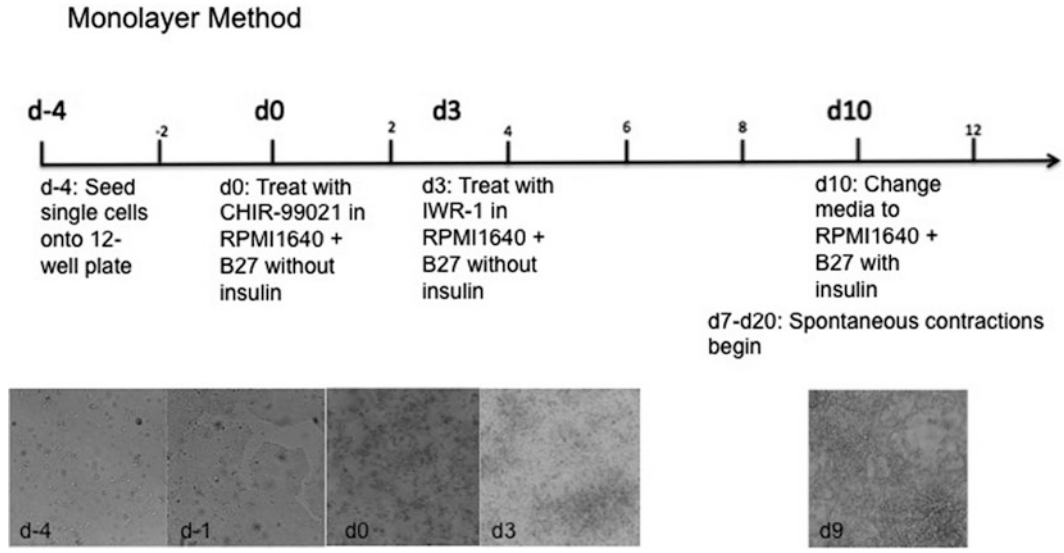

Fig. 2 Timeline of monolayer method from day −4 to day 10 with corresponding light microscope images taken at 4× magnification

13. Significant cell death will be observed after 24 h. If there are no cells attached to the plate, the cells had not reached appropriate confluence at day 0. Consider waiting an additional day for the cells to reach 100 % confluence before treatment.
14. Change in morphology can be observed, with the cells creating a transparent, weblike pattern. Cell death will continue to be observed while differentiation occurs.
15. Beating can be expected to begin from around day 7 to about 2 weeks after the treatment with CHIR-99021.

A summary of the major steps of the protocol is given in Fig. 2.

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Generation and Characterization of Patient-Specific iPSC Model for Cardiovascular Disease

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Abstract

Advances in differentiation of cardiomyocytes from human induced pluripotent stem cell (hiPSC) were emerged as a tool for modeling of cardiovascular disease that recapitulates the phenotype for the purpose of drug screening, biomarker discovery, and testing of single-nucleotide polymorphism (SNP) as a modifier for disease stratification. Here, we describe the (1) retroviral reprogramming strategies in the generation of human iPSC, (2) methodology in characterization of iPSC in order to identify the stem cell clones with the best quality, and (3) protocol of cardiac differentiation by modulation of Wnt signaling and β-catenin pathway.

Keywords: Human induced pluripotent stem cell (hiPSC), Retroviral reprogramming, Stem cell characterization, Cardiac differentiation, Cardiovascular disease modeling

1 Introduction

Pluripotent cells, such as embryonic stem cells, are invaluable tools for research and able to potentially serve as a source of cell- and tissue-replacement therapy. Rejection after transplantation of cells and tissue derived from embryonic stem cells is a significant obstacle to their clinical use. Instead of using rodent animal for drug screening or disease modeling study, stem cell can be used as a human platform to test for drug efficacy and potential adverse effect and to study pathogenesis of disease in different organs. Recently, human somatic cells have been reprogrammed directly to pluripotent by ectopic expression of four transcription factors, named as induced pluripotent stem cell (hiPSC) [1–6]. The viral based introduction of stem cell-inducible factors is the most common method because of its high reproducibility, high efficiency, and the convenience of quality control.

Yee Ki Lee, X. Ran, and K. W. H. Lai are the co-first authors.

In this chapter, based on our previous experiences in human iPSC generation and cardiac disease modeling [7–13], we describe the methodology from human iPSC cell generation, characterization, and cardiac cell differentiation for cardiovascular diseases modeling. We use constitutively active retroviral vectors that stably integrated into the host cell genome to introduce c-Myc, Klf4, Oct4, and SOX2. The generated hiPSC will be subjected to a series of stem cell characterization experiments. Firstly, iPS cells can be characterized by a high level of alkaline phosphatase (AP) expression which, along with the expression of surface markers including stage-specific embryonic antigens, SSEA-4, tumor-related antigen (TRA), Tra1-60 and -81, and pluripotency transcription factors, Oct4 and Nanog, indicates a subset of cells with self-renewal potential. In normal circumstances, the retroviral transgenes are usually silenced toward the end of reprogramming [14]; we next perform the Oct4, SOX2, Myc, and Klf-4 transgene silencing assay by RT-PCR to confirm that the reprogrammed cells are able to overcome the hurdle of partially reprogrammed with dependency on exogenous gene by stimulation endogenous expression of stem cell transcription factor induced by the transgenes. Further experiment will be carried out to study the activation of endogenous OCT4 gene expression by bisulfite genomic sequencing analysis that evaluates demethylation statuses of cytosine guanine dinucleotides (CpG) in OCT4 promoter regions. The success in transgene silencing of newly reprogrammed cell population will allow us to identify stable clone for consistent differentiation of stem cell for disease modeling or generate enough amount of target cell for regenerative purposes. Next, karyotyping analysis is performed to check the genome stability and reduce the risk of picking up tumorigenic clone that contains abnormal number of chromosome, translocation of loci, deletion of certain regions, or conversion that may affect cell cycle progression. To investigate whether the generated hiPSC are capable of self-renewal and of potential to differentiate in vitro and in vivo into a wide variety of cell types, including derivatives of all three embryonic germ layers, which are usually assessed as nestin (ectoderm), intercellular adhesion molecule 1 (ICAM1), and troponin-I (mesoderm), we usually perform in vitro differentiation by embryoid bodies (EBs) plating in serum medium or teratoma formation assay in SCID mice. Once the iPSCs are characterized, it will be followed by using β-catenin-Wnt signaling pathway manipulation to drive cardiac differentiation [15] for cardiac disease modeling.

2 Materials

2.1 Components for iPSC Generation

2.1.1 Chemicals

1. VPA (100 mM): Dissolve 0.17 g Valproic acid sodium salt (Sigma-Aldrich) in 10 ml PBS and filter by 0.22 μm syringe filter.
2. Gelatin (0.1 %): Dissolve 5 g of gelatin powder (Sigma) in 500 ml DPBS and autoclave.
3. Mitomycin C (2 mg/ml): Dissolve 2 mg in PBS without $Ca^{2+}Mg^{2+}$ and filter.
4. Calcium phosphate kit: 2 M $CaCl_2$, 2× HEPES-buffered saline (HBS), cell culture-grade water (Life Technologies).
5. Polybrene: 1 mg/ml (Millipore).

2.1.2 Vectors

1. Four retroviral vectors, pMXs coding for pluripotent inducible factors, hOCT3/4, hSOX-2, hMyc, and hKlf4 in addition to FCQ pMM2-eGFP as a GFP-positive control were obtained from Addgene.

2.1.3 Medium

1. 293T/CF-1 medium: High-glucose DMEM, 10 % FBS (Gibco), 4 mM L-glutamine, NEAA, penicillin.
2. iPSC medium: Knockout DMEM, 20 % KOSR (Gibco), 8 ng/μl bFGF, 230 μl β-mercaptoethanol (55 mM), NEAA, 1 % penicillin–streptomycin.
3. Defined FBS (DFBS) medium: Knockout DMEM (Gibco), 20 % FBS (Catalogue number: SH30700, Hyclone), 4 mM L-glutamine, 8 ng/μl bFGF, 100 U/ml 15 penicillin–streptomycin.
4. Induction medium: 1:1 iPSC medium and defined FBS medium.
5. mTeSR medium (Stem Cell Technologies).
6. DMEM-F12 (Invitrogen).

2.1.4 Cell Line

1. 293T (ATCC).
2. CF-1 (ATCC) (isolation protocol as shown in Sections 3.1.1–3.1.3).

2.2 iPSC Characterization

2.2.1 Components for Stem Cell Marker Immunofluorescence Staining

Reagents

1. Ca^{2+}/Mg^{2+}-free phospho-buffered saline (Thermo-Fisher).
2. PBS-T wash buffer: DPBS with 0.1 % Triton X-100 (Sigma-Aldrich).
3. Tween 20 (Affymetrix).
4. 4 % paraformaldehyde (MSD).
5. Dispase (Invitrogen).
6. Matrigel (Corning).
7. Y27632 (Sigma-Aldrich).

Table 1
List of antibodies for immunofluorescence staining of stem cell markers

Primary antibody[a]	Host species	Secondary antibody[a]	Conjugate
Oct 4	Rabbit IgG	Goat anti-rabbit IgG (H + L) secondary antibody	Alexa Fluor® 488 conjugate
SSEA-4	Mouse IgG_3	Goat anti-mouse IgG (H + L) secondary antibody	Alexa Fluor® 594 conjugate
Nanog	Rabbit IgG	Goat anti-rabbit IgG (H + L) secondary antibody	Alexa Fluor® 488 conjugate
TRA-1-60	Mouse IgM	Goat anti-mouse IgM heavy-chain secondary antibody	Alexa Fluor® 594 conjugate
Sox2	Rabbit IgG	Goat anti-rabbit IgG (H + L) secondary antibody	Alexa Fluor® 488 conjugate
TRA-1-81	Mouse $IgM_{,k}$	Goat anti-mouse IgM heavy-chain secondary antibody	Alexa Fluor® 594 conjugate

[a]The primary antibodies are purchased from Stemgent (Cambridge, M.A.) and the secondary antibodies are obtained from Molecular Probes, Life Technologies

Antibodies

1. Stem cell marker antibodies for co-stain (Table 1).
2. Slow Fade Gold® antifade reagent with DAPI (Life Technologies).

2.2.2 Components for Alkaline Phosphatase

Reagent Preparation

1. Add 5 μl of Tween 20 into 10 ml of 1× PBS to make a final concentration of 0.05 % PBST.
2. Alkaline Phosphatase Staining Kit (Stemgent): Mix Solution A and Solution B, and then incubate at room temperature for 2 min. Add Solution C to the Solution A and B mixture. The ration of Solution A, Solution B, and Solution C should be 1:1:1.

2.2.3 Components for Transgene Silencing

1. Primers used in this study are shown in Table 2.
2. TRI Reagent (Applied Biosystems).
3. Quantitect®Reverse transcription (RT) kit (Qiagen).
4. AmpliTaq Gold 360 Tag polymerase mix (Applied Biosystems).

2.2.4 Components for Karyotyping Analysis

Reagent Preparation

1. Mitotic inhibitor solution: Demecolcine solution (10 mg/ml) (Sigma).
2. Dye solution: Mix 7 ml of Giemsa with 100 ml of distilled water and filter.
3. Hypotonic solution: 75 mM KCl.
4. Fixative solution: Methanol/glacial acetic acid 3:1 (BDH, AR grade or equivalent).

Table 2
Primers used for transgene silencing assay by RT-PCR

Gene	Direction	Sequence (5′–3′)
Endo OCT3/4	Forward	CTT CCC TCC AAC CAG TTG CCC CAA AC
	Reverse	GAC AGG GGG AGG GGA GGA GCT AGG
Endo SOX2	Forward	GGG AAA TGG GAG GGG TGC AAA AGA GG
	Reverse	TTG CGT GAG TGT GGA TGG GAT TGG TG
Endo KLF4	Forward	ACG ATC GTG GCC CCG GAA AAG GAC C
	Reverse	TGA TTG TAG TGC TTT CTG GCT GGG CTC C
Endo MYC	Forward	GCG TCC TGG GAA GGG AGA TCC GGA GC
	Reverse	TTG AGG GGC ATC GTC GCG GGA GGC TG
pMXs-L3205	Reverse	CCC TTT TTC TGG AGA CTA AAT AAA
Exo OCT3/4	Forward	CCC CAG GGC CCC ATT TTG GTA CC
Exo SOX2	Forward	GGC ACC CCT GGC ATG GCT CTT GGC TC
Exo KLF4	Forward	ACG ATC GTG GCC CCG GAA AAG GAC C
Exo MYC	Forward	CAA CAA CCG AAA ATG CAC CAG CCC CAG
GAPDH	Forward	AGC CAC ATC GCT CAG ACA CC
	Reverse	GTA CTC AGC GCC AGC ATC G

5. Carnoy's solution: 1 g of ferric chloride ($FeCl_3$) dissolved in 24 ml of absolute alcohol, 12 ml of chloroform, and 4 ml of glacial acetic acid.
6. 20× SSC: Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml water. Adjust the pH to 7.0 with HCl. Adjust the volume to 1 l with ultrapure water. Dispense into aliquots. Sterilize by autoclaving.

2.2.5 Components for OCT4 Promoter Demethylation Analysis

1. EZ DNA Methylation™ Kit (Zymo Research). Storage: The CT Conversion Reagent supplied within this kit is a solid mixture and must be prepared prior to first use.

Components for DNA Bisulfide Conversion

1. CT Conversion Reagent: Add 750 μl water and 210 μl of M-Dilution Buffer to a tube of CT Conversion Reagent and mix at room temperature with frequent vortexing or shaking for 10 min. If not used immediately, the CT Conversion Reagent solution can be stored overnight at room temperature, 1 week at 4 °C, or up to 1 month at −20 °C. The CT Conversion Reagent is light sensitive, so minimize its exposure to light. For the best results, the CT Conversion Reagent should be used immediately following preparation. Stored CT Conversion Reagent solution must be warmed to 37 °C.
2. M-Binding Buffer: Add 24 ml of 100 % ethanol to the 6 ml M-Wash Buffer concentrate (D5001) before use.

Table 3
List of antibodies for three germ layer staining of in vitro and in vivo (teratoma) differentiation

Primary antibody[a]	Host species	Secondary antibody[a]	Conjugate
Endoderm: Alpha Fetoprotein	Mouse	Rabbit anti-mouse IgG (H + L) secondary antibody	Alexa Fluor® 594 conjugate
Mesoderm: Smooth muscle actin	Mouse	Rabbit anti-mouse IgG (H + L)	Alexa Fluor® 594 conjugate or HRP conjugate
Ectoderm: Nestin	Rabbit	Goat anti-rabbit IgG (H + L)	Alexa Fluor® 488 conjugate

[a]Primary and secondary antibodies are obtained from Millipore and Life Technologies, respectively

3. Genomic DNA extraction kit: QiAamp® DNA Mini Kit (Qiagen)
4. Amplitaq gold 360 (Applied Biosystems)
5. PCR clean-up kit: Wizard® SV Gel and PCR Clean-Up System (Promega)

2.2.6 Three Germ Layer Formation Assay

1. TRIPLES-EXPRESS (Gibco).
2. F12-DMEM (Gibco).
3. 4 % Buffered formalin solution (Sigma).
4. Characterization antibodies kit for three germ layer marker (Chemicon). Details of antibodies are listed in Table 3.

2.3 Cardiac Differentiation Components

2.3.1 Chemicals and Reagents

1. Y27632 (5 mM): In a sterile environment, add 6.24 ml of DMSO to 10 mg of Y27632. Divide the solution into 100 µl aliquots in 1.5 ml tubes and store them at −20 °C for up to 1 year.
2. GSK-β inhibitor: CHIR99021 (36 mM): Add 1.49 ml of DMSO to 25 mg of CHIR99021 and store at −20 °C for up to 1 year.
3. IWP2 (5 mM): Add 4.28 ml of DMSO to 10 mg of IWP2. Incubate the mixture at 37 °C for 10 min to dissolve the IWP2. Divide the solution into 100 µl aliquots in 1.5 ml tubes and store them at −20 °C for up to 1 year.
4. Accutase (Gibco).

2.3.2 Medium

1. mTeSR1 + 5 mM Y27632: Add 50 µl of 5 mM Y27632 to 50 ml of mTeSR1 (the final concentration of Y27632 is 5 µM). Store the solution at 4 °C for up to 2 weeks.
2. RPMI/B-27 (510 ml): Mix 500 ml of RPMI and 10 ml of B-27 supplement. The medium can be stored at 4 °C for up to 1 month.

Table 4
Panel of antibodies used for cardiac cell characterization by immunofluorescence staining

Primary antibody	Company	Host species	Secondary antibody[a]	Conjugate
Troponin T	Thermo	Mouse	Rabbit anti-mouse IgG (H + L)	Alexa Fluor® 488 conjugate
Tropomyosin	Sigma			
α-Actinin	Sigma			

[a]Secondary antibodies were obtained from Life Technologies

3. RPMI/B-27 without insulin (510 ml): Mix 500 ml RPMI and 10 ml of B-27 without insulin. The medium can be stored at 4 °C for up to 1 month.
4. RPMI/B-27 without insulin + 12 μM CHIR99021 (24 ml): Add 8 μl of 36 mM CHIR99021 into 24 ml of RPMI/B-27 without insulin. (*Freshly make and not recommend storing this medium.*)

2.3.3 Antibodies for Cardiac Characterization

Details for antibodies staining for cardiac-specific markers are showed in Table 4.

3 Methods

3.1 iPSC Generation

All steps are carried out in a tissue culture hood under aseptic conditions.

3.1.1 Isolation of CF1 Mouse Embryonic Fibroblast (CF1-MEF)

1. A pregnant CF1 mouse at 13–15 d.p.c. (day post-coitum) was sacrificed by cervical dislocation and its uterine horns were dissected out and rinsed by sterile PBS. Normally 13–15 embryos are obtained from single pregnant CF1 mouse (Fig. 1a).
2. Embryos were dissected out from the placenta and embryonic sac (Fig. 1b).
3. The head and the internal organs (red color) of embryo were removed under dissection microscope (Fig. 1c, d).
4. The dissected embryos were transferred to a new petri dish and rinsed by sterile PBS.
5. Then the embryos were minced into less than 1 mm^3 by sterile surgical blade.
6. Minced tissue was collected in 50 ml Falcon tube. PBS was removed and 10 ml of 0.5 % trypsin solution was added, and incubated in 37 °C water bath with occasional shaking for 10 min.

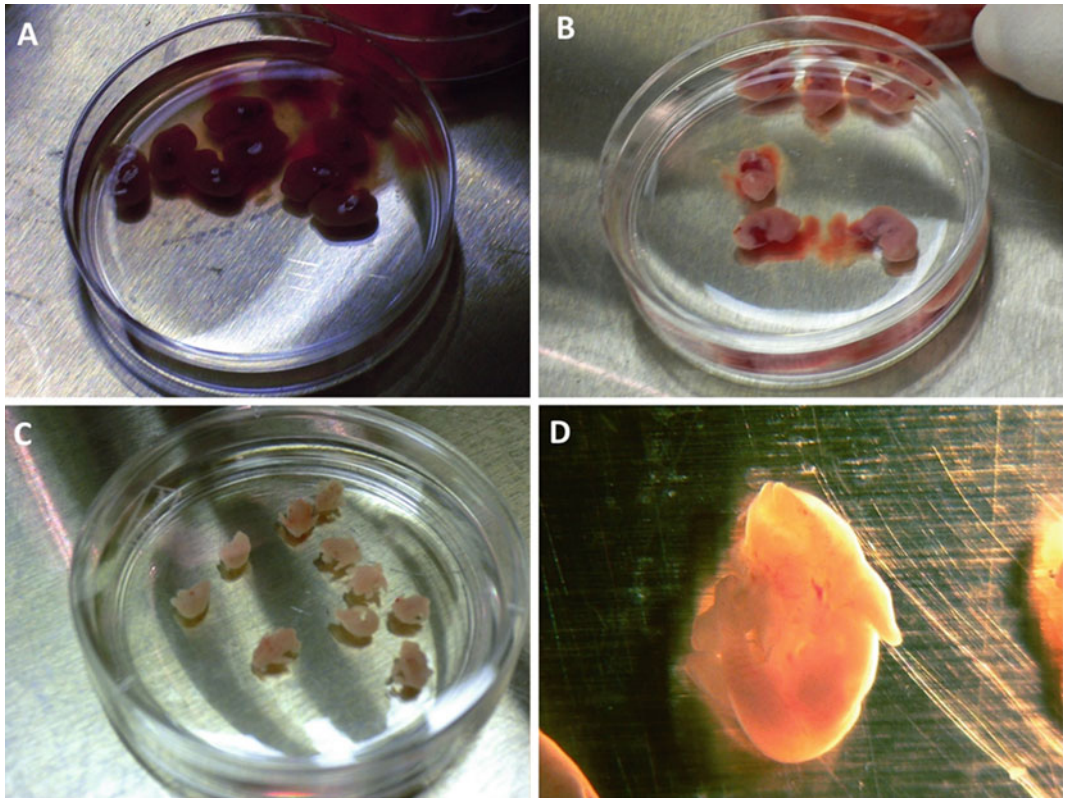

Fig. 1 Preparation of CF1 mouse embryos for MEF generation. (**a**) Embryos were extracted from the uterine horns. (**b**) Internal organs are shown in *red color* after removal of embryonic sac. (**c** and **d**) Head and internal organs are removed and the embryos are ready for MEF preparation

7. The supernatant was collected and neutralized by MEF culture medium, and then 10 ml of new 0.5 % trypsin solution was added into the tube and incubated as **step 6**.
8. Collect the supernatant and add trypsin again. **Steps 6** and **7** were repeated until most of the tissue was digested.
9. The neutralized supernatant was centrifuged at 400 × *g* for 5 min to collect the cell pellet.
10. Wash the CF1-MEF twice by MEF medium and resuspend the cell pellet in 10 ml MEF medium.
11. Plate the cells derived from about to three to four embryos in each T175 (TPP) flask. The cells were harvested at ~80 % confluent (usually 24–48 h) by 0.05 % TE and can be frozen for future use (this is the passage 0 (P0) of the CF1-MEF).
12. Expand the remaining T175 flask(s) of P0 cells till P3 or P4, and then inactivate the cell proliferation by either gamma radiation or mitomycin-C treatment.

3.1.2 Inactivation of MEF by Mitomycin-C Treatment

1. Coat 6-well plate with 0.1 % gelatin and incubate at RT for at least 2 h.
2. Aspirate medium from MEFs and wash with PBS without $Ca^{2+}Mg^{2+}$.
3. Place 20 ml of medium containing 20 μg/ml of mitomycin C on MEFs.
4. Incubate for 2 h at 37 °C with mitomycin C-containing medium, then wash twice with PBS, trypsinize, centrifuge (for 5 min at 300 × *g*), and resuspend cells in warm medium.
5. Count cells and plate at a density of 15,000–18,000 cells/cm^2 in each well of gelatin-coated 6-well plate. It would be ready for stem cell culture on the other day, or the inactivated cells can be frozen for later uses.

3.1.3 Inactivation of MEF by Gamma Irradiation

1. Coat 6-well plate with 0.1 % gelatin and incubate at RT for at least 2 h.
2. Wash the MEF in T175 by PBS without $Ca^{2+}Mg^{2+}$ and then dissociated by 5 ml of 0.05 % TE solution at 37 °C for 5 min.
3. Collect the dissociated MEF and wash twice by CF1 medium.
4. Resuspend the cell in 5 ml of CF1 medium and ready for gamma irradiation.
5. Put the tube of cells into the sample can of the gamma irradiator.
6. Set the time of irradiation to 3500 rad.
7. After irradiation, centrifuge the cell at 800 × *g* for 5 min and then seed onto the gelatin-coated plate. The remaining cells could be frozen for later use.

3.1.4 Retroviral Packaging

1. Thaw one vial of 293T cells to a 10 mm dish 5 days before infection.
2. After 24 h, change 293T medium on the 100 mm dish.
3. Split 4×10^6 293T cells into five 100 mm culture dishes for each different plasmid (one plate for FCQ-pMM2-eGFP, one for Sox2, one for Klf4, one for Oct4, one for cMyc); the cell cultures are expected to have around 70 % confluency on the day of transfection.
4. On the day after, change around 7.5 ml 293T medium to each dish gently.

5. Prepare 5 × 15 ml conical tubes for each different plasmid: pMXs-GFP, pMXs-hSOX2, pMXs-hKLF4, pMXs-hOct4, and pMXs-hcMyc. Mix 20 μg PLC with 20 μg either one of the pMXs vector or GFP, sterilize water to make up a volume of 1250 μl, and then further add 156.25 μl 2 M $CaCl_2$ and 1250 μl 2× HBS in order to make up a final volume of 2.5 ml transfection cocktail. Once HBS is added, pipette the mixture for 20–30 times vigorously and count for 2 min.
6. Transfer the transfection cocktail to 293T cells and incubate overnight.
7. Replace the transfection medium with 10 ml of fresh 293T medium after 12–16-h transfection.
8. Carefully collect the viral supernatant, and add another 10 ml 293T medium to the 293T cells for second infection.
9. Filter the viral supernatant using 0.45 μm syringe. Mix the filtered viral supernatant with polybrene (4 μg/ml) before infection. Proceed to Section 3.1.5 for transduction of donor cells (Fig. 2).

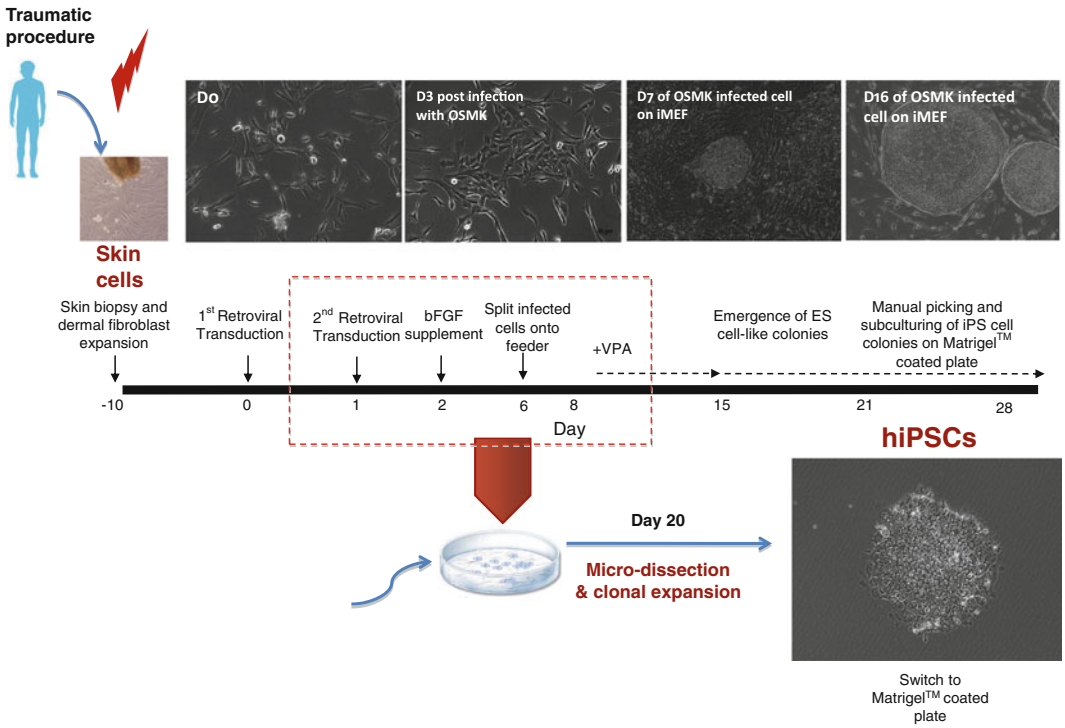

Fig. 2 Workflow of hiPSC generation by retroviral reprogramming of skin fibroblast. Morphology change was observed 3 days after Oct4, SOX2, Myc, and Klf4 infection. Embryonic stem cell-like colonies were observed on feeder culture after 16 days of infection

10. Carefully collect the virus supernatant after another 24-h transfection as described in **step 6**. Repeat the transduction step according to Section 3.1.5.

3.1.5 Retrovirus Infection and iPSC Generation

1. Day −10 (afternoon): Preparing donor cells for infection 1 day before infection. Use 0.05 % (wt/vol) trypsin-EDTA to trypsinize donor cells. Add basal medium to neutralize trypsin activity. Transfer donor cells to a 15 ml conical tube, and pellet cells by centrifugation at 200 × *g* for 4 min. Then count the cells and seed onto 6-well plate (50,000–100,000/well).
2. Day 0: Transfer 2 ml of each virus supernatant (Oct4 + SOX2 + Klf4 + Myc) or GFP containing 4 μg/ml polybrene to a single well of the donor cells with 2 ml medium.
3. Day 1 (morning): After 12–14 h, discard the culture medium, and refresh with the donor cell medium.
4. Day 1 (afternoon): Repeat **step 2** to perform the second-round infection.
5. Day 2 (morning): Aspirate the culture medium and refresh the donor cells with DFBS medium at 12 h after the second infection.
6. Days 3–5: Keep on refreshing infected donor cell with DFBS medium.
7. Till morphology change of infected cell can be observed, prepare irradiated mouse embryonic fibroblast (MEF) feeder cells 1 or 2 days before donor cells split on feeder. Seed 1.2×10^6 IRR-MEF on each gelatin-coated 100 mm dish.
8. Day 6: Wash the MEF feeder with plain DMEM/F12 once, and add 10 ml induction medium. Wash the SKOM cells with PBS once, trypsinize them using 0.05 % (wt/vol) trypsin-EDTA, and incubate at 37 °C for 3 min. Add DFBS medium to neutralize trypsin activity. Seed 5×10^3 to 1×10^4 of infected cell into 10 cm dish.
9. Days 8–15: Refresh with induction medium containing 1 mM VPA daily.
10. Human ESC-like colonies will start to appear on day 15. Replace the medium with hESC medium (if on feeder)/ mTesR1 medium (if on feeder free).
11. Manually pick the cell colonies to feeder-free culture since day 20.

3.2 iPSC Characterization

3.2.1 Pluripotent Marker Immunofluorescence Analysis

Preparation of iPSC for Pluripotent Marker Immunofluorescence Analysis

1. On the day of splitting, the 6-well iPSC culture should have 70–85 % confluence.
2. Prepare Matrigel-coated 24-well plates with 12 mm glass cover slide. Place the Matrigel-coated plated at room temperature for 15 min before use.
3. Rinse each 6-well iPSC culture with DMEM-F12, aspirate, and then add 1 ml of 1 mg/ml dispase per well and incubate for 4–6 min.
4. Aspirate dispase, and rinse each well twice with DMEM-F12.
5. Add 1 ml mTeSR1 medium and then use the cell scrapers to scrap the cell off the surface of the culture vessel.
6. Collect the cell suspension from each well into 15 ml conical tubes, and centrifuge at 200 × *g* for 3 min.
7. Aspirate the supernatant and resuspend the cell pellet with mTeSR1 medium and 5 μM Y27632.
8. Aspirate the Matrigel from the Matrigel-coated plate. Transfer the cell suspension into each Matrigel-coated well.
9. Incubate the cells in a 37 °C in humidified atmosphere of 5 % CO_2 overnight.
10. On the next day, replace the medium with pre-warm mTeSR1 medium. Repeat this medium replacement daily until cells are 50–70 % confluent.

Immunostaining

1. Fix the cells in 4 % paraformaldehyde for 15 min at room temperature.
2. Wash the cell three times with PBST wash buffer on the orbital shaker for 5 min. Repeat three times.
3. Incubate cells with primary antibody (1:200) overnight at 4 °C. In order to verify stem cell characteristic of the generated iPSC, OCT4 (1:100) was co-stained with SSEA-4 (1:100), and Nanog (1:100) was stained together with Tra1-60 (1:100) and SOX-2 (1:100) vs. Tra1-81 (1:100) [7, 10].
4. Rinse the cells three times for 5 min with PBST wash buffer.
5. Incubate cells with secondary antibody (1:500) for 1 h at room temperature.
6. Further wash three times.
7. Counterstain and mount with Slow Fade Gold® antifade reagent with DAPI.
8. The detail information of antibodies used in current study is shown in Table 1. Images were acquired using Carl Zeiss fluorescence microscope with AxioVision 6.0 software or fluorescence confocal microscope Carl Zeiss LSM 700 (Zeiss GmbH, Gottingen, Germany). Examples for stem cell marker staining are shown in Fig. 3.

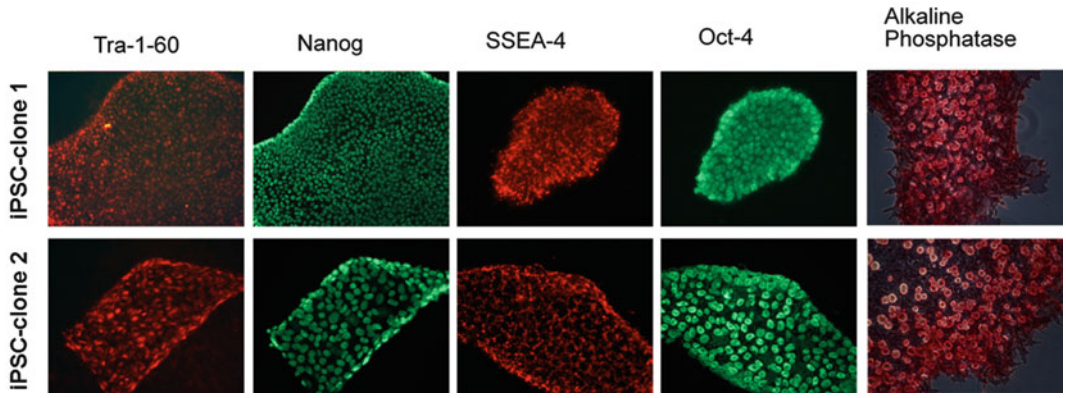

Fig. 3 Immunofluorescence staining of stem cell markers. Tra1-60 was co-stained with Nanog and SSEA-4 was co-stained with Oct4. High expression of alkaline phosphatase (AP) confirmed the feature of stem cell in the generated hiPSC

Alkaline Phosphatase Staining

1. Discard the medium and rinse the cells with 0.5 ml 1× PBST.
2. Fix the cells with 1 ml fix solution for 2–5 min at room temperature.
3. Discard 1× PBST and add 0.6 ml freshly prepared AP Substrate Solution.
4. Wrap the cell culture with foil and incubate for 5–15 min at room temperature.
5. Aspirate the AP Substrate Solution to stop the reaction.
6. Rinse the cells twice with 1× PBS.
7. Counterstain and mount with Slow Fade Gold® antifade reagent with DAPI.

3.2.2 Transgene Silencing

1. Total RNA from cells at various stages was extracted with TRI® reagent. And the RNA amount is quantified by Nanodrop 2000 nucleic acid detection system.
2. Use 1 μg RNA for reverse transcription to perform reverse transcription with QuantiTect® reverse transcription kit (Qiagen) according to the manufacturer's instruction.
3. Prepare PCR mix with AmpliTaq Gold 360 Taq polymerase mix and run the PCR cycle GeneAmp PCR system 9700 (both from Applied Biosystems) at pre-denaturation at 95 °C for 5 min (denaturation at 95 °C for 30 s, annealing at 57–60 °C for 30 s, and extension at 72 °C for 90 s) for 30 cycles followed by post-extension step at 72 °C for 10 min.

4. Primers sequences of the four exogenous reprogramming transgenes (pMXs-hOCt3/4, -hSOX-2, hMyc, and hKlf-4) and endogenous hOCt3/4, -hSOX-2, hMyc, and hKlf-4 plus Nanog were depicted in the previous study [1]. GAPDH was used as an internal control for RT efficiency and RNA integrity.
5. Run the PCR reaction mix with 2 % agarose gel.
6. Measure the gene expression levels by band intensity.

3.2.3 Karyotype Analysis

1. Place slides in a beaker containing 6 M HCl for at least 3 h at room temperature. After that, wash slides in running tap water for 10 min. Leave the slides immersed for at least one night in 100 % ethanol and then rinse and store in distilled water at room temperature (before **step 11**).
2. 80–90 % confluent iPSC cultures were arrested at metaphase by exposing to colcemid (100 μg/ml) for 4 h (*see* **Note 1**).
3. The cells were washed by F12-DMEM and dissociated by 1 ml of TRIPLES-EXPRESS in each of the 6-well.
4. Incubate the cells at 37 °C for desirable time till the cells start to round up (*see* **Note 2**).
5. Wash the cell once with F12-DMEM and harvest in stem cell culture medium.
6. Centrifuge for 10 min at 1600 rpm.
7. Aspirate the supernatant and resuspend the pellet in 6 ml of hypotonic solution.
8. Incubate at room temperature for 15 min and centrifuge.
9. Aspirate the supernatant and resuspend the pellet in 6 ml of fixative solution.
10. Leave at room temperature for 5 min and store in the refrigerator at 4 °C for at least one night.
11. Centrifuge the fixed samples.
12. Aspirate the supernatant with vacuum pump and resuspend, by vortexing, with 0.5–2 ml of Carnoy's fixative according to the size of pellet obtained.
13. Dispense few drops of cell suspension on a glass slide on water bath and spread the material on the slide with a glass pipette.
14. Fixed material by quickly passing slide on the Bunsen flame and leave the slides at room temperature.
15. Put the dried slides in 1× SSC equilibrated at +60 °C and add 0.2 N HCl acid up to the grinding of the slide. Incubate for 5 min.
16. Transfer the pairs of slides from the jar removed from the thermostat to 60 °C to distilled water, and then rinse under

with tap water in the jar and emptying it. Repeat at least five times.

17. Dry the slides at room temperature for at least 2 h.
18. Put the pairs of slides into 100 ml of staining solution, and then set time which can vary from 2 to 15 min (*see* **Note 3**).
19. Rinse the slides with running water.
20. At this point the slides are allowed to air-dry. And check on the quality of staining with an optical microscope (*see* **Note 4**).
21. Mount slide hydrophobic mountant with a cover slip.

3.2.4 OCT4 Promoter Demethylation Analysis

1. Exact genomic DNA from parental somatic cell line, such as dermal fibroblast and iPSC.
2. The genomic DNA (1 μg) is subjected to CT conversion using EZ DNA methylation kit (Zymo Research, Orange, CA, USA), according to the manufacturer's instructions.
3. Prepare 1 μg genomic DNA in nuclease-free water with total volume of 45 μl.
4. Add 5 μl of M-Dilution Buffer to the DNA sample. Mix the sample by flicking or pipetting up and down.
5. Incubate the sample at 37 °C for 15 min.
6. After the above incubation, add 100 μl of the prepared CT Conversion Reagent to each sample and mix. Incubate the sample in the dark at 50 °C for 12–16 h.
7. Incubate the sample at 0–4 °C (e.g., on ice) for 10 min.
8. Add 400 μl of M-Binding Buffer to a Zymo-Spin™ IC Column and place the column into a provided collection tube.
9. Load the sample (~150 μl from **step 6**) into the Zymo-Spin™ IC Column containing the M-Binding Buffer. Close the cap and mix by inverting the column several times.
10. Centrifuge at full speed (>10,000 × *g*) for 30 s. Discard the flow-through.
11. Add 100 μl of M-Wash Buffer to the column. Centrifuge at full speed for 30 s.
12. Add 200 μl of M-Desulphonation Buffer to the column and let stand at room temperature (20–30 °C) for 15–20 min. After the incubation, centrifuge at full speed for 30 s.
13. Add 200 μl of M-Wash Buffer to the column. Centrifuge at full speed for 30 s. Add another 200 μl of M-Wash Buffer and centrifuge for an additional 30 s.
14. Place the column into a 1.5 ml microcentrifuge tube. Add 10 μl of M-Elution Buffer directly to the column matrix. Centrifuge for 30 s at full speed to elute the DNA.

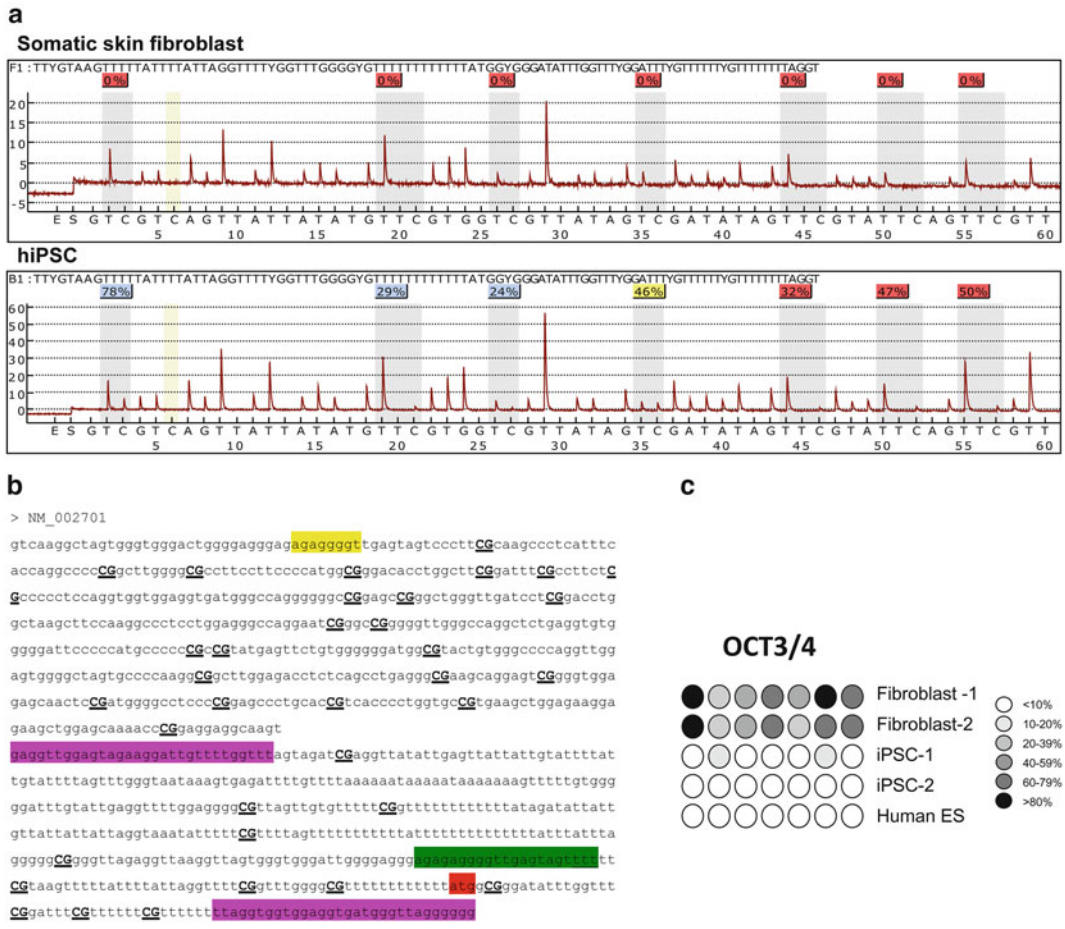

Fig. 4 Oct4 promoter demethylation in iPSC. (**a**) As indicated by the results of pyrosequencing, somatic skin fibroblast cell showed a highly methylated status (0 % unmethylated leading to low CT conversion rate) in Oct4 promoter, which indicated a silencing of the gene expression. CpG region containing 24–78 % unmethylated rate in Oct promoter region of hiPSC, which showed an activation of the gene expression. (**b**) The region of promoter amplified for bisulfide sequencing analysis of OCT4 promoter methylation status. *Underline* indicates the methylation sites of our interest. CG sequence *underlined* indicates the position subjected to DNA methylation. *Pink*: PCR amplification primers with the reverse primer biotinylated; *green*: forward sequencing primer; *red*: start codon; describe pyrosequencing and interpretation of the data. (**c**) Typical presentation of OCT4 promoter methylation status. Seven methylation sites were included in the region of analysis. Each *circle* represents one CG site which may be subjected to methylation. *iPSC* induced pluripotent stem cell, *human ES* human embryonic stem cell

15. By using 4 μl the CT-converted DNA samples, amplify the promoter regions of the human OCT4 gene using forward primer: 5′ GAGGTTGGAGTAGAAGGATTGTTTTGGTTT 3′ and the biotinylated reverse primer: 5′ CCCCCCTAACC-CATCACCTCCACCACCTAA 3′ with the product size of 467 bp. The PCR condition: (95 °C for 30 s, 60 °C for 30 s and 72 °C for 90 s) × 35 cycles (Fig. 4b).

16. Run 2 % agarose gel electrophoresis to confirm the specificity of 5 μl of PCR products, and then perform PCR cleanup of PCR product with 100 μl elution volume.
17. 50 μl of PCR products are sequenced using Pyrosequencing PSQ96 HS System (Biotage, Uppsala, Sweden) with the sequencing primer (20 μM), 5′ AGAGAGGGGTTGAGTAGTTTT 3′. The methylation status of each locus was analyzed using PyroQ-CpG software (Qiagen) with an example showed in Fig. 4a, c.

3.2.5 Three Germ Layer Formation Assay by In Vitro Differentiation and Teratoma Formation Assay

1. Coat glass cover slips with enough gelatin solution to cover the surface of glass.
2. Incubate at 37 °C for 30 min.
3. Micro-dissect the contracting outgrowths from day 21 differentiated hiPSC-embryoid bodies plated in 20 % FBS medium using a glass knife.
4. Add 1 ml of 0.25 % (wt/vol) trypsin-EDTA at 37 °C for 5 min.
5. Dissociate the outgrowths into individual cells and small clusters, by using pipette up and down.
6. Add 20 % FBS medium to neutralize trypsin activity. Transfer the cell suspension into 15 ml Falcons, and centrifuge at 200 × *g* for 3 min.
7. Aspirate the supernatant, and do not disturb the pellet.
8. Resuspend the cells with 20 % FBS medium.
9. Plate the cells on cover slips coated with 0.1 % gelatin solution.
10. Incubate cells overnight at 37 °C in humidified atmosphere of 5 % CO_2.
11. Stain the cells with the protocol same as in Section 3.2.1, **step 2.** Representative photos are showed in Fig. 5a.

Teratoma Formation with Three Germ Layer

1. iPSCs were dissociated into single cells by 1 ml of TRIPLES-EXPRESS in every 6-well for 5 min.
2. The dissociated iPSCs were washed by 5 ml mTesR1 medium and resuspended in F12-DMEM at 1×10^7 cells per ml.
3. 0.1 ml iPSCs were injected subcutaneously at the neck region of non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (i.e., 1×10^6 cells per site).
4. After 5–7 weeks, tumors were resected, fixed in 4 % buffered formalin, and then processed for paraffin sectioning (Fig. 5b).
5. Immunohistological staining of the three germ layers was then preformed using the human embryonic germ layer characterization kit and examination of structure with fetoprotein as the endoderm marker, smooth muscle actin as the mesoderm marker, and MAP as the ectoderm marker (Fig. 5c).

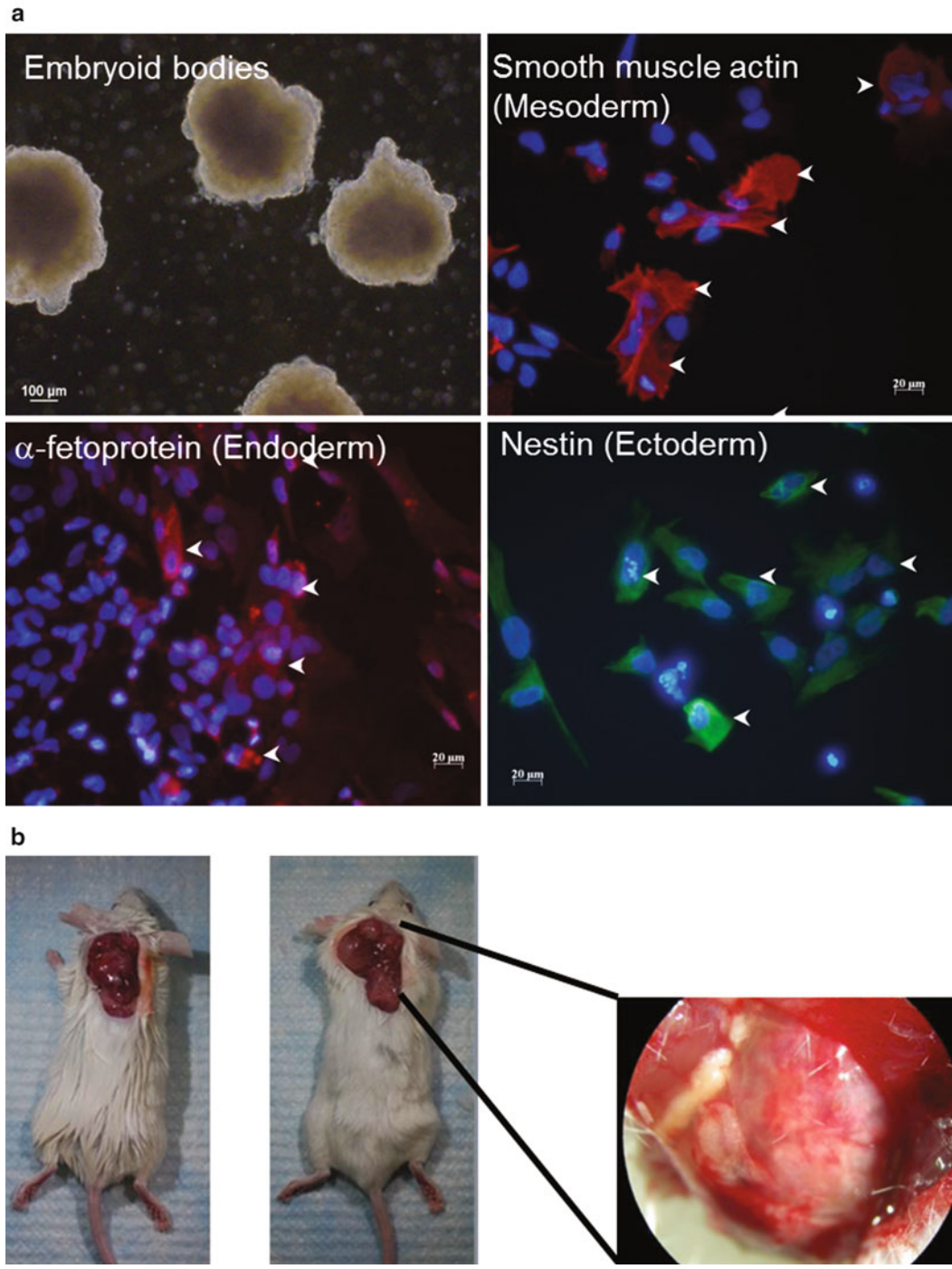

Fig. 5 In vitro and in vivo differentiation of hiPSC forming three germ layers. (**a**) Differentiation of embryoid bodies forming cells derived from mesoderm (indicated smooth muscle actin positive stain), ectoderm (indicated by nestin positive stain), and endoderm (alpha-fetoprotein). (**b**) Human iPS cell-induced teratoma formation 5 weeks after subcutaneous injection in NOD/SCID mice and (**c**) subsequently stained for three germ layer markers (reproduced from Lai et al. [8]; permission from Mary Ann Liebert, Inc. does not required for the author of the manuscript for reuse in this book chapter)

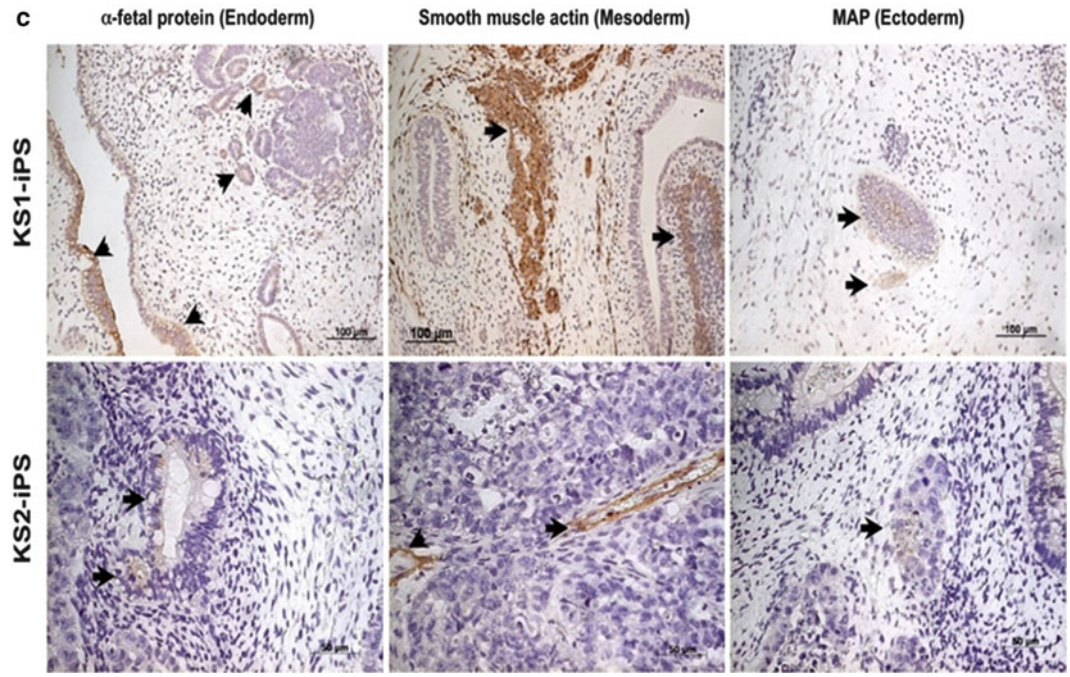

Fig. 5 (continued)

3.3 Generation of hiPSC-Derived Cardiac Cell Model (Workflow Is Showed in Fig. 6a)

3.3.1 Cardiac Differentiation

1. Add 0.5 ml diluted Matrigel (dilution factor according to the packing insert from the manufacturer) per well of 12-well plate for at least 2 h at 4 °C.
2. Remove culture medium from iPS cells. Add 1 ml per well DMEM/F12 to rinse the cells and remove the medium.
3. Add 1 ml of Accutase (Gibco) to each well. Incubate at 37 °C, 5 % CO_2, and wait for 8 min or you can observe that the iPSC is round up.
4. Add 1 ml of mTeSR1 into each Accutase-treated well and pool all of the cells in a 15 ml Falcon. Centrifuge the cells at 200 × *g* for 5 min at room temperature. Discard the supernatant and resuspend the cells by mTeSR1 medium with 5 μM Y27632. Count the total cell number with a hemocytometer.
5. Seed the iPSC onto the Matrigel-coated 12-well plate at 0.5, 0.75, 1.0, 1.25, or 1.5 million cells per well in order to optimize for suitable cell density for cardiac differentiation. Different cell lines may be various (*see* **Note 5**).
6. Incubate at 37 °C for 24 h and then change with 2 ml fresh mTeSR1 medium.
7. Keep changing the medium for 3 more days with mTeSR1 medium.

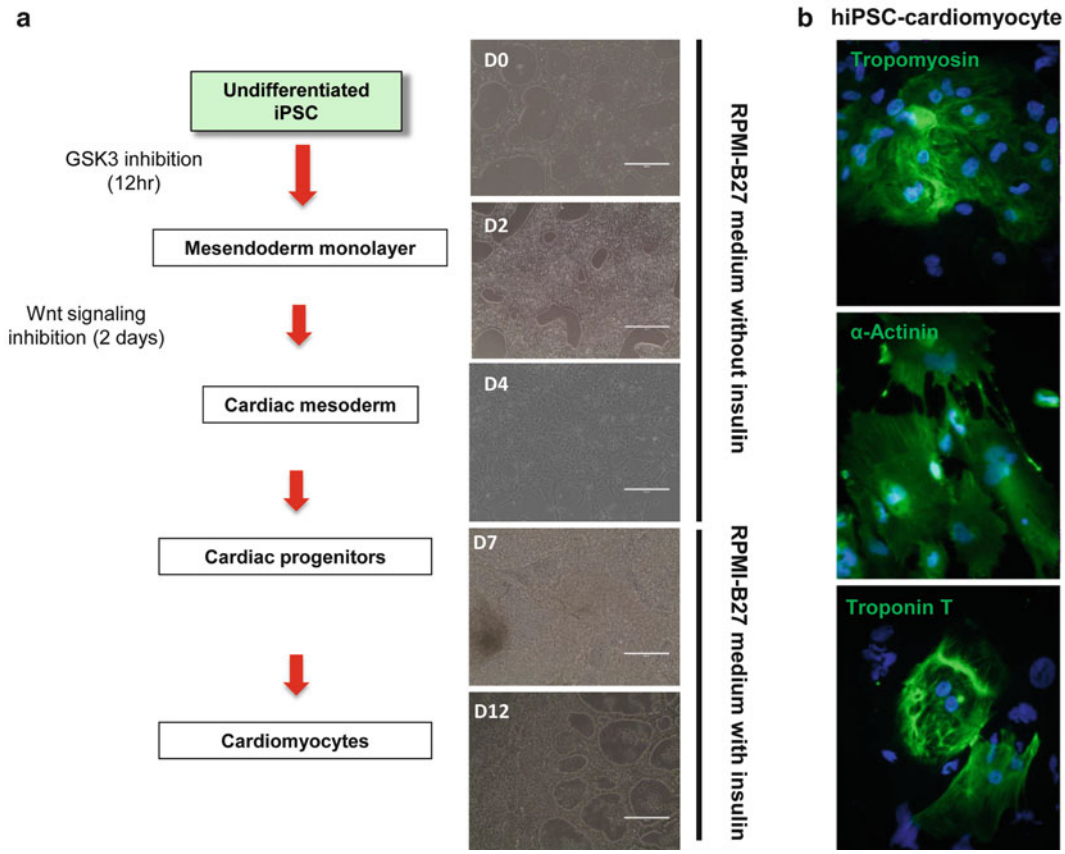

Fig. 6 (**a**) Workflow of cardiomyocytes differentiation from hiPSC by Wnt/beta-catenin signaling modulation; (**b**) cardiac-specific troponin structure of human hiPSC-derived cardiomyocyte was revealed by tropomyosin and troponin-T staining and alpha-actinin was served as another cardiac marker

At day 4, remove the mTeSR1 medium, add 2 ml of RPMI/B-27 without insulin + 12 μM CHIR99021, and incubate at 37 °C for exactly 24 h (*see* **Notes 6** and 7).

8. Remove the medium and add 2 ml of RPMI/B-27 without insulin for 48 h.
9. Collect the medium and mix with equal volume of fresh RPMI/B-27 without *insulin* to have a combined medium.
10. Filter the combined medium, add 1 μl/ml of stock IWP2 (final concentration at 5 μM), and then add 2 ml per well to the differentiating cells.
11. 48 h later, change with 2 ml of RPMI/B-27 without insulin and culture for 3 days.
12. Change with RPMI/B27 medium for every 2–3 days. Beating cluster or cells will appear 5–7 days later.

3.3.2 Isolation of hiPSC-Derived Cardiomyocytes

1. Coat glass cover slips with enough gelatin solution to cover the surface of glass.
2. Incubate at 37 °C for 30 min.
3. Micro-dissect the contracting outgrowths from day 21 differentiated hiPSC-embryoid bodies using a glass knife.
4. Beating cardiomyocytes were dissociated with 0.25 % (wt/vol) trypsin-EDTA at 37 °C for 5 min.
5. Dissociate the outgrowths into individual cells and small clusters, by using pipette up and down.
6. Add 20 % FBS medium to neutralize trypsin activity. Transfer the cell suspension into 15 ml Falcons, and centrifuge at 200 × *g* for 3 min.
7. Aspirate the supernatant, and do not disturb the pellet.
8. Resuspend the cells with 20 % FBS medium.
9. Plate the cells on cover slips coated with 0.1 % gelatin solution.
10. Incubate cells overnight at 37 °C in humidified atmosphere of 5 % CO_2.
11. Proceed to immunostaining step according to the protocol described in Section "Immunostaining." Typical staining of cardiac marker is showed in Fig. 6b.

4 Notes

1. It is important to make sure that a single-cell suspension is achieved to obtain good metaphase spreads.
2. After hypotonic and fixative treatment in a glass tube, the cell suspension was spread on slides, and chromosomal analysis was performed on Giemsa-banded metaphases using a bright-field microscope.
3. They are immersed in the Giemsa stain solution for a time that varies from a minimum of 15 min to a maximum of 24 h. In fact the staining time varies a lot depending on the temperature and humidity in the laboratory; to overcome this problem a first group of slides is colored by setting a time allegedly under the terms "climate" present that day; according to the quality of the coloring it is to determine the staining time of the second group of slides.
4. In the event that problems occur in coloring (mitosis little or too colored), the slides can be decolorized in a solution composed of 50 % of absolute ethanol and 50 % distilled water for about 5–10 min and left to dry for at least half an hour and staining is repeated.
5. The starting seeding cell density is crucial for efficient cardiac differentiation. The initial plating density and/or the time of

expansion before initiation of differentiation may require optimization for different cell lines or expansion conditions.

6. We recommend plating at a cell density of 0.5 million cells/well of 12-well plate, increasing this stepwise to 1.5 million for your specific hPSC lines in your first experiment, and then expanding the cells for 4 days before initiation of differentiation. Once you have identified the optimal seeding density for your specific hPSC lines, you can use this seeding density for subsequent differentiation experiments.
7. Record the time when you added RPMI/B-27 without insulin with CHIR99021 is necessary, as the medium has to be changed at exactly 24 h later. Although we identified 12 μM CHIR99021 as the optimal concentration for the six lines that we tested, other lines may respond to CHIR99021 treatment differently. Thus, titration of CHIR99021 concentrations may be required. We recommend the dose for testing at 6–14 μM CHIR99021.

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Transgene-Free Disease-Specific iPSC Generation from Fibroblasts and Peripheral Blood Mononuclear Cells

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Abstract

Induced pluripotent stem cells (iPSCs) offer great promise as tools for basic biomedical research, disease modeling, and drug screening. In this chapter, we describe the generation of patient-specific, transgene-free iPSCs from skin biopsies and peripheral blood mononuclear cells through electroporation of episomal vectors and growth under two different culture conditions. The resulting iPSC lines are characterized with respect to pluripotency marker expression through immunostaining, tested for transgene integration by PCR, and assayed for differentiation capacity via teratoma formation.

Keywords: Induced pluripotent stem cells, iPSC, Disease modeling, Transgene-free, Episomal vector, Characterization of pluripotency

1 Introduction

The process of transcription factor-mediated somatic cell reprogramming discovered by Shinya Yamanaka has made possible the generation of pluripotent stem cells from a wide range of adult differentiated cells including human skin cells [1]. This process results in induced pluripotent stem cells (iPSCs) that are remarkably similar to embryonic stem cells with respect to self-renewal and differentiation ability. As virtually any cell type from any individual can be reprogrammed, it has become possible to create patient- and disease-specific stem cells [2]. Such custom-made stem cells are genetically identical to the donors, and therefore, are great tools for studying genetic basis of diseases, for drug screening and development of genotype-specific therapeutics, and, in the future, for the production of rejection-free cells for transplantation-based therapies. As a testament to the rapid adoption of the reprogramming technology, large numbers of iPSC-based models of monogenic and complex diseases have been generated [3].

Originally, the reprogramming process involved the delivery, and stable expression of four transcription factors (Oct4, Sox2, Klf4, and c-Myc) into the target adult cell populations via

retro- or lentiviral vectors. While the efficiency of reprogramming with such vectors are relatively high, these vectors are mutagenic due to their integration into the host genome. Alternative means of expressing the four reprogramming factors have emerged that avoid or minimize the risk of damage to the genome of the recipient cells, such as transfection of mRNAs, non-integrating viral vectors and episomal DNA vectors [4]. While each of these methods have unique advantages and disadvantages, in this protocol, we will be focusing on the generation of iPSCs using episomal plasmids, mainly because of their ease of use and consistent efficiency of reprogramming [5, 6]. In addition, episomal plasmids can be used to derive iPSC lines form skin fibroblasts and peripheral blood cells, both of which are readily accessible patient cell types [7]. Furthermore, with the advent of iPSC culturing techniques that do not require mitotically inactivated mouse embryo fibroblast (MEF) feeders or undefined medium components, it is now possibly to routinely generate patient-specific iPSC lines in a xeno-free manner [8, 9]. In this chapter, we outline a strategy that combines electroporation of episomal DNA vectors with a feeder-free culture method to derive patient-specific iPSCs.

Once iPSC lines are established, a number of validation experiments are carried to demonstrate that the lines express pluripotency markers, are devoid of genomic integration, and are capable of differentiation into derivates of the three germ layers. Expression of pluripotency markers such as Oct4, Nanog, SSEA4, Tra-1-60, and Tra-1-81 can be tested by RT-PCR or by immunofluorescence staining. To demonstrate that the resulting iPSC lines do not contain any integrated episomal vector sequences carrying the reprogramming factors, a PCR-based detection method from genomic DNA is utilized. Finally, to test for the multi-lineage differentiation potential, iPSC lines are subjected to the teratoma formation analysis, which is at present the gold standard for human pluripotent cell lines [10, 11].

2 Materials

Prepare all mediums under a laminal flow hood.

2.1 Cell Culture Materials

1. Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium (catalog number 14190-235, Gibco)
2. Collagenase IV (catalog number 17104-019, Gibco)
3. Ficoll-Paque (catalog number 17-1440-02, GE Healthcare)
4. Rock Inhibitor (Y-27632) Dissolve in DMSO at a final concentration of 10 mM (1000×). Aliquot and store at −80 °C.
5. Matrigel (Corning)

6. Irradiated CF-1 MEFs (catalog number GSC-6001G, GlobalStem)
7. *0.1 % Gelatin*: Dissolve 0.1 % Gelatin (catalog number G1890, Sigma) in distilled water, mix, and autoclave. Store at room temperature.
8. *Vitronectin* (*VTN*): Thaw the vitronectin (catalog number A14700, Gibco), aliquot into microcentrifuge tubes in small volumes (such as 60 μl), and store at −80 °C.
9. *EDTA dissociation solution* (*EDTA/PBS*): Add 500 μl EDTA (0.5 M) (Ultra pure EDTA pH 8.0 catalog number 15575-038, Invitrogen) to 500 ml calcium- and magnesium-free PBS. Add 0.9 g NaCl and adjust osmolarity to 340 mOsm. Filter through a 0.22 μM filter and store at +4 °C for up to 6 months.
10. *Reprogramming plasmids*: pCXLE-hOCT3/4-shp53-F (Addgene plasmid 27077), pCXLE-hUL (Addgene plasmid 27080), pCXLE-SK (Addgene plasmid 27078), pCXWB-EBNA1 (Addgene plasmid 37624) (*see* **Note 1**).

2.2 Cell Culture Media

1. Fibroblast culture medium (D10): Combine all reagents at indicated ratios: 89 % DMEM (with L-glutamine and pyruvate), 10 % fetal bovine serum (FBS), 1 % penicillin/streptomycin solution (100×). Mix the components and filter through with 0.22 μm filter. Store at +4 °C up to 4 weeks.
2. Human embryonic stem cell medium (hESC medium): Make up a 0.1 % bovine serum albumin (BSA) solution in sterile PBS. Dissolve bFGF at 10 μg/ml in 0.1 % BSA/PBS to prepare a 1000× stock solution. Aliquot into microcentrifuge tubes and store at −20 °C. Combine all reagents at indicated ratios: 77.8 % DMEM/F12 (catalog number 11330-057, Gibco), 20 % Knock-out Serum Replacement (KOSR, catalog number 10828-028, Gibco), 1 % nonessential amino acids (MEM-NEAA), 1 % L-glutamine, 1 % penicillin/streptomycin, 0.1 mM 2-mercaptoethanol (2-ME), 10 ng/ml bFGF. Mix the components and filter through 0.22 μm filter. Store at +4 °C for up to 2 weeks (*see* **Note 2**).
3. E8 base medium: Combine all components at indicated final concentrations in DMEM/F12: 64 mg/l L-ascorbic acid, 13.6 μg/l sodium selenite, 1 g/l NaCl. Adjust the pH to 7.4 by adding NaOH.
4. Complete E8 medium: Thaw 500 ml E8 base medium and combine the following reagents with E8 base medium at the following final concentrations: 10 μg/ml holo-transferin, 100 μg/ml bFGF, 1.74 ng/ml TGF-β1, 20 ng/ml insulin. Filter through 0.22 μm filter. Store at +4 °C for up to 2 weeks (*see* **Note 2**).

5. Reprogramming E8: Prepare reprogramming medium with the same formula as full E8 medium but leave out the TGF-β1. Instead, add sodium butyrate at a final concentration of 0.1 mM.
6. StemSpan H3000 medium with cytokines: Thaw the H3000 medium (Catalog number 09800, Stem Cell Technologies) and add cytokines at the indicated concentrations: 100 ng/ml IL-6, 300 ng/ml SCF, 300 ng/ml TPO, 300 ng/ml Flt3 ligand, 10 ng/ml IL-3.
7. mTeSR1 Medium (catalog number 5850, Stem Cell Technologies).
8. E8 cryopreservation (freezing) medium: Add 2 ml of sterile DMSO to 8 ml Complete E8 medium. Store at +4 °C for up to 1 week.
9. hESC cryopreservation (freezing) medium: Combine all reagents at indicated ratios: 50 % FBS, 40 % hESC medium, and 10 % DMSO. Filter through 0.22 μm DMSO-safe filter. Store at +4 °C for up to 1 week.

2.3 Antibodies for Immunostaining

Alexa Flour-555 mouse anti-SSEA4 (560218, BD Biosciences), Alexa Flour-488 mouse anti-human Tra-1-81 (560174, BD Biosciences), Oct4 antibody (ab19857, Abcam), Nanog antibody (ab21624, Abcam), Biotin conjugated anti-human Tra-1-60 (330604, Biolegend), secondary Alexa Fluor 488 goat anti-rabbit IgG (A11008, Invitrogen), secondary streptavidin-HRP secondary antibody (405210, Biolegend).

3 Methods

The starting cell source for iPSC generation can be dermal fibroblasts or peripheral blood mononuclear cells (PBMCs).

3.1 Establishing Primary Cultures of Fibroblasts from Patient Samples

A 3 mm skin punch biopsy should be obtained by a physician. Prior to the procedure, the patient must have given informed consent under a protocol approved by the relevant Institutional Review Board. The biopsy is placed in fibroblast culture medium (D10 medium) in a 15 ml Falcon tube and transported to the laboratory on ice.

1. By working under a laminar flow biosafety cabinet, remove the excess D10 medium present in the Falcon tube that contains the biopsy. Add 10 ml of DPBS (without calcium and magnesium) to completely saturate and wash the biopsy twice with DPBS.
2. By utilizing sterile forceps, transfer the biopsy to a sterile 6-well plate. Using a pair of sterile razor blades, cut the biopsy into 0.5–1 mm pieces, and put a sterile cover slip on two or three

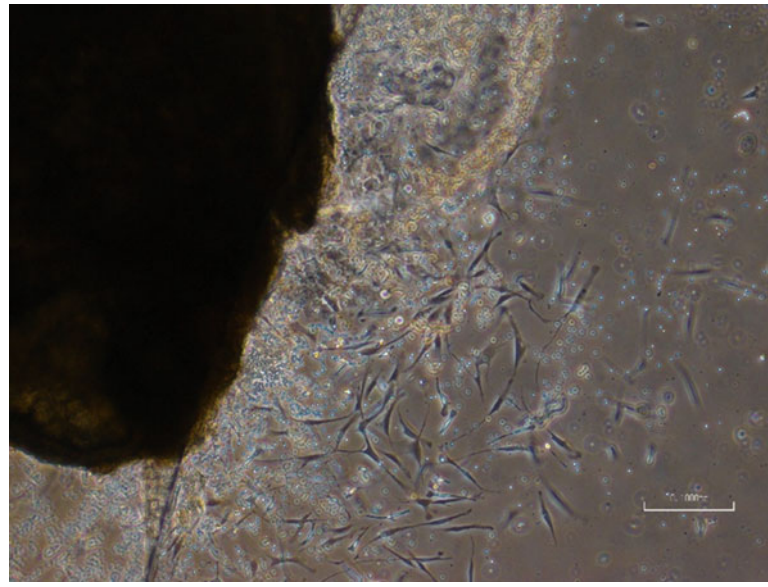

Fig. 1 Fibroblast outgrowth from a skin punch biopsy piece 11 days after initiation of culture. In some cases emergence of fibroblasts may be preceded by the outgrowth of keratinocytes which are observed as epithelial sheets and not as individual cells

small pieces of biopsy. Slowly add 2 ml primary culture medium into the well taking care not to dislodge the biopsy pieces. Carefully place the plate into a 37 °C, 5 % CO_2 incubator.

3. After 3 days, remove the medium and add fresh D10 medium to the wells. After the initial replacement of the medium, check fibroblast cultures daily for growth. Replace medium every 3 days until fibroblast outgrowth is observed and cell lines are established, which could take up to 3 weeks (Fig. 1).
4. When explant growth has covered ~75–90 % of the culture area of the well, passage the cells by removing the medium; wash once with DPBS, add 0.05 % trypsin/EDTA solution, and incubate at 37 °C to allow cells to detach for 5–7 min (*see* **Note 3**). Add D10 medium directly into the wells to inactivate the trypsin, gently pipet up and down to make a homogenous suspension, and split at a ratio of 1:3 to new plates. Allow the fibroblasts to grow to 80–90 % confluence. Once the cells have become confluent passage cells as above (*see* **Note 4**).

3.2 Cryopreservation of Skin Fibroblast Lines

1. Prepare fibroblast freezing medium by mixing D10 medium (50 %), FBS (40 %), and DMSO (10 %). Filter the freezing medium through a 0.22 μm DMSO-safe filter. In order to freeze fibroblast grown on a 10 cm dish, wash once with 6 ml PBS, add 2 ml of 0.05 % trypsin/EDTA solution, and incubate

for 3–5 min at 37 °C. To inactivate the trypsin add 4 ml fibroblast culture medium to the dish and gently pipet the mixture up and down to make a homogenous suspension.

2. Add the cell suspension to a 15 ml Falcon tube and centrifuge 5 min at 1200 rpm. During the centrifugation, label two cryogenic vials with the name, date, and passage number of the cells.
3. Aspirate the supernatant and resuspend the cell pellet in 2 ml freezing medium. Aliquot 1 ml to each of two cryogenic vials. Place vials into a cryogenic carrier (e.g., Mr. Frosty, Thermo Scientific) and transfer to a −80 °C freezer. Next day, move the vials into the vapor phase of a liquid nitrogen tank.
4. To thaw a patient-derived fibroblast cell line, take the cryogenic vial from liquid nitrogen, quickly put in a 37 °C water bath, and shake gently. When thawing is complete, add 1 ml of D10 medium into the cryogenic vial and combine the contents with 4 ml of D10 medium in a 15 ml Falcon. Centrifuge for 5 min at 1200 rpm. Aspirate and resuspend the cell pellet in 4 ml D10 medium. Pipet up and down to make a homogenous suspension and transfer onto a 10 cm culturing dish. Add 8 ml D10 medium and allow for cells to attach to the vessel.

3.3 Introduction of the Reprogramming Factors into Fibroblasts by Electroporation

For electroporation we use the Neon Transfection system (Life Technologies). Other systems such as the Amaxa nucleofector (Lonza) can also be used.

1. Prepare the Neon transfection system by filling the electroporation cuvette with 3 ml of electrolyte buffer E2.
2. For each electroporation, combine 1.2 μg of each of the three plasmids encoding the reprogramming factors (pCXLE-hOCT3/4-shp53-F, pCXLE-hUL, pCXLE-hSK) with 120 μl resuspension buffer R in a 1.5 ml microcentrifuge tube. Total plasmid amount will be 3.6 μg in 120 μl R buffer. Mix by pipetting (*see* **Note 5**).
3. When patient fibroblasts reach 80 % confluency (Fig. 2a), trypsinize and count the cell numbers using a hemocytometer. Each electroporation requires 3×10^5 cells. For each electroporation, take 3.6×10^5 cells and centrifuge at 1200 rpm for 5 min at room temperature.
4. Discard the supernatant and resuspend in the 120 μl R buffer/plasmid solution and mix gently by pipetting. Using 100 μl tip of Neon transfection kit, carefully take 100 μl cell, R buffer, and plasmid mixture into the Neon tip and insert the tip into cuvette.
5. Set the parameters of electroporator to 1400 V, 20 ms, 2 pulse, and push start on the screen (*see* **Note 6**). After the

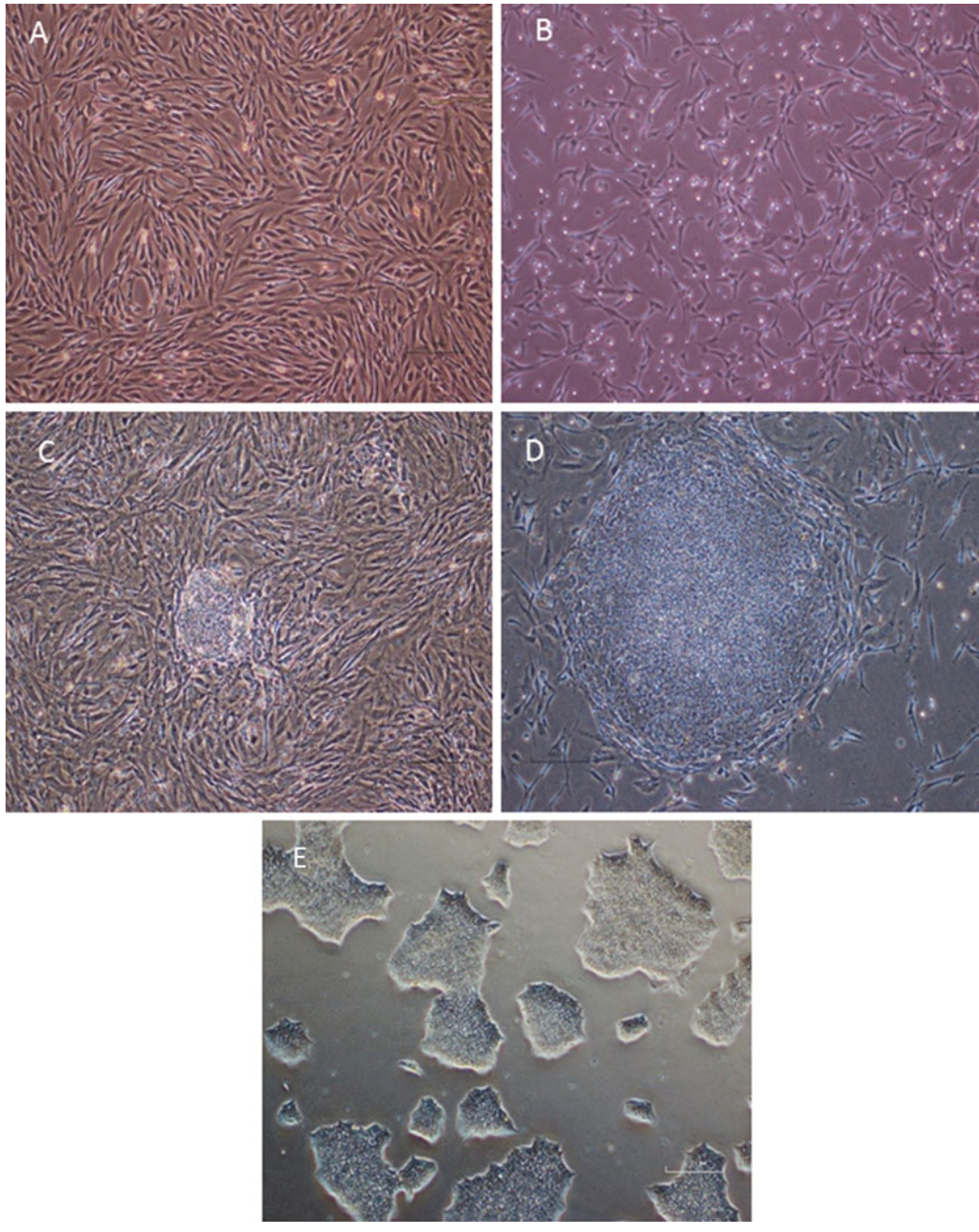

Fig. 2 Fibroblasts and the emergence of iPSC colonies during the reprogramming process. (**a**) Patient-derived cultures of skin fibroblasts. (**b**) One day after electroporation of fibroblasts most cells attach to the plate, but note that there are also a number of floating dead cells. (**c**) A small cluster of iPSCs among the MEF layer 14 days after electroporation of fibroblasts. (**d**) An iPSC colony at passage 1 on MEFs. (**e**) Established iPSC colonies in E8/VTN culture conditions

electroporation is complete, empty the contents of the tip into one well of a 6-well plate which contains 2 ml D10 medium without antibiotics. The presence of antibiotics in the medium right after electroporation decreases cell viability. Shake the plate to mix cells in the well and incubate 37 °C 5 % CO_2. This is considered as day 0 of reprogramming.

6. On day 1, change the medium with D10 medium containing antibiotics (Fig. 2b). Optional: To increase reprogramming efficiency, add 3 μM Dot1L inhibitor (EPZ004777 (iDot1L)) to the medium until day 14 [12].
7. On day 7, passage the cells onto either (a) mitotically inactivated MEFs to be grown in hESC medium or (b) onto vitronectin (VTN)-coated tissue culture plates to be grown in E8 medium. VTN/E8 combination results in feeder-free derivation of iPSCs in a chemically defined medium.

3.4 Preparation of Plates with Inactivated MEFs (Should Be Performed on Day 6)

1. Coat the cell culture dishes with 0.1 % gelatin and incubate at room temperature for 30 min.
2. Thaw a vial of inactivated MEFs (mitotically arrested either by irradiation or mitomycin C treatment) by holding the cryogenic vial in a 37 °C water bath until only a small amount of ice is left. Immediately but slowly add 1 ml of warm D10 media by drop, mix, and transfer to Falcon tube with 10 ml D10 medium. Spin at 1200 rpm for 5 min.
3. During centrifugation, aspirate gelatin and add 1 ml D10 medium per 10 cm^2 (one well of a 6-well plate). Resuspend MEF pellet in an appropriate volume so that the resulting cell concentration is 1.5–2 × 10^5 cells/ml. Plate 1 ml per 10 cm^2. Transfer to incubator and shake plate back/forth, left/right to ensure even distribution.
4. On day 7, trypsinize the electroporated human fibroblasts and seed onto MEF-seeded 6-well plates in D10 medium (*see* **Note 7**). Next day, replace the D10 medium with hESC medium. Replace hESC medium every 2 days until day 14 and thereafter every day until colonies appear (Fig. 2c).

3.5 Preparation of Vitronectin-Coated Tissue Culture Plate (Should Be Performed on Day 7)

1. Thaw an aliquot of VTN and dilute in PBS to 0.5 μg/ml. Coat the tissue culture plates with 1 ml/10 cm^2. Shake the plate gently to cover all surfaces and incubate at room temperature for 1 h. Prior to use aspirate the VTN solution (*see* **Note 8**).
2. Trypsinize the electroporated human patient fibroblasts and seed onto VTN-coated plates in D10 medium at a 1:4 to 1:8 ratio. Next day, change the medium to E8 reprogramming medium and continue feeding the cells daily until day 14. Replace the E8 reprogramming medium with full E8 medium

on day 14 and re-feed the cells thereafter every day until colonies appear.

3.6 Isolation and Manual Passaging of iPSC Colonies

When the emerging iPSC colonies are big enough, manually dissect and passage them onto new MEF-coated plates with hESC medium or onto VTN-coated plates with E8 medium (Fig. 2d).

1. 2 h before colony picking, add 1 ml hESC medium or E8 onto reprogramming plates to reduce stress and to increase cell viability. Mark each colony to be picked under an inverted light microscope with a permanent marker.
2. Place a stereomicroscope into a laminar flow hood and locate the marked colony. Using a 10 μl pipette tip, divide the colony into small pieces such that each piece contain approximately 50 cells. Transfer the all the pieces of one colony into a separate well.
3. Do not disturb the plate for the next 48 h to allow for attachment of iPSC clusters.
4. After 48 h change the medium and keep feeding daily until the clusters become big enough to be passaged again, which usually takes 5–7 days.

3.7 Enzymatic Passage and Cryopreservation of iPSCs Grown on MEFs

Once 10–15 large colonies are present within a well, the entire well can be passaged enzymatically.

1. Thaw and plate MEFs 1 day prior to passaging as in Section 3.4.
2. Eliminate any differentiated colonies in the well to be passaged by removing them with a pipette tip under the stereomicroscope in a laminar flow hood. Differentiated colonies will have undefined boundaries and may contain vertical growth areas that appear as brown regions.
3. Aspirate the medium and wash with 1 ml DMEM/F12 per 10 cm^2 and add 1 ml of Collagenase IV/10 cm^2 (diluted to 1 mg/ml in DMEM/F12). Incubate at 37 °C for 5 min. Aspirate the Collagenase IV and add 1 ml DMEM/F12.
4. Scrape the cells by using a cell lifter and collect the colonies in a falcon tube. Add 1 ml DMEM/F12 onto the same well to collect remaining iPSC colonies and combine in the same falcon tube. Centrifuge at 1000 rpm for 2 min.
5. During centrifuge, aspirate D10 medium from fresh MEF-coated plates and add hESC medium.
6. Typical splitting ratio is 1:4; therefore for one well of a 6-well plate, when centrifugation is completed, aspirate the supernatant and resuspend the cell pellet in 2 ml hESC medium. Mix gently by pipetting for a few times to break the colonies into even-sized clusters and dispense 0.5 ml onto each new well

(*see* **Note 9**). Place the plate into the incubator and shake well to distribute iPSC clusters. Feed the cells daily with hESC medium until they are ready to be passaged again, which should be in 4–6 days.

7. To cryopreserve iPSCs grown on MEFs prepare hESC Cryopreservation (freezing) medium as indicated in Section 2.2.
8. Follow the same procedure for splitting the iPSC colonies until **step 4**. After collecting the colonies from one well and centrifugation, resuspend in 0.5 ml cold hESC freezing medium and place in to a cryogenic vial. Make sure that the vial is labeled clearly with the name of the iPSC line, passage number, and date. We generally freeze the contents of a well of a 6-well plate into a single vial. Place the vials into a cryogenic carrier at −80 °C. One day later transfer into the vapor phase of a liquid nitrogen tank.

3.8 Passaging and Cryopreservation of iPSCs Grown on VTN-Coated Plates

1. Aspirate the medium and wash once with PBS and add 1 ml EDTA dissociation solution per 10 cm^2. Incubate for 3 min at 37 °C, 5 % CO_2
2. Aspirate the EDTA dissociation solution and quickly add 2 ml fresh E8 medium. Lift and disperse the colonies by forceful pipetting. We generally split the cells grown on VTN with E8 medium at a 1:8 ratio; therefore transfer 400 μl of the cell suspension into a new VTN-coated well. It is important to work quickly as cells will attach rapidly. Shake the plates to evenly distribute the cells and move into the incubator. Replace the medium daily with complete E8 medium (Fig. 2e).
3. To cryopreserve iPSC lines grown on VTN with E8 medium, follow the same procedure for passaging the cells until **step 3**. Collect the cells in a falcon tube and centrifuge at 1000 rpm for 2 min and then resuspend in E8 cryopreservation medium. We generally freeze one confluent well of a 6-well plate into two cryogenic vials.

3.9 Generation of iPSCs from Human Peripheral Blood Mononuclear Cells

1. 10 ml of blood is taken by venipuncture by a qualified health care personnel into an EDTA tube and tube is inverted a few times. Mix 10 ml of blood with 10 ml PBS in a 50 ml falcon tube making sure that the volumes are 1:1.
2. Use two 15 ml Falcon tubes for the PBMC isolation. Add 5 ml Ficoll-Paque to each falcon tube and gently layer 10 ml of blood/PBS mixture on top of it making sure that the two layers do not mix. Spin the tubes at 400 × *g* for 30 min at 18 °C. Set the centrifuge to slow acceleration and no breaks.
3. Remove the plasma from the top fraction without disrupting the mononuclear cells at the interface. The PBMC layer should appear as an off-white layer on top of the centrifuged Ficoll.

Gently collect the PBMCs at the interface by using serologic pipettes and place into a new 15 ml tube and then add 12 ml of PBS. Spin at 200 × *g* for 10 min at 18 °C without brake.

4. Resuspend the cell pellet in 3 ml of H3000 medium (without cytokines) and count with a hemocytometer. Prepare aliquots of 3×10^6 cells in 1.5 ml tubes and spin at 200 × *g* for 10 min at 18 °C without brake.
5. Resuspend the cells in the H3000 medium containing cytokines plate 3×10^6 cells in 2 ml medium into a well of a 6-well plate. Incubate at 37 °C, 5 % CO_2, for 6 days. Medium change is not needed.
6. On day 5 after the initial isolation of PBMCs, which is 1 day prior to the electroporation of the reprogramming factors, prepare 6-well plates with inactivated MEFs as explained in Section 3.5. Next day replace the medium of MEF-coated plates with H3000 medium with cytokines and keep in the incubator. Prepare the Neon transfection system by filling the electroporation tube with 3 ml of electrolytic buffer E2 and place it into the electroporation cuvette.
7. Collect the floating PBMCs in the H3000 medium into 15 ml Falcon tube and count the cells (*see* **Note 10**). Spin at 200 × *g* for 10 min at 18 °C without brake. Aspirate the supernatant and resuspend the pellet in 240 μl Buffer T.
8. For each electroporation, combine 1.2 μg of each of the three plasmids encoding the reprogramming factors (pCXLE-hOCT3/4-shp53-F, pCXLE-hUL, pCXLE-hSK) and the pCXWB-EBNA1 plasmid with 120 μl resuspension buffer T in a 1.5 ml microcentrifuge tube such that the total amount of plasmid DNA will be 4.8 μg (*see* **Note 11**). Mix by pipetting. Using 100 μl tip of Neon transfection kit, carefully take up 100 μl cell, T buffer, and plasmid mixture into the Neon tip and insert the tip into cuvette.
9. Set the parameters of electoporator to 2150 V, 20 ms, 1 pulse, and push start on the screen. After the electroporation is complete, seed the cells onto the MEF-coated plates containing H3000 medium with cytokines and 3 μM Dot1L inhibitor (iDot1L). On days 8, 10, and 12 add 1.5 ml of mTeSR1 medium onto the wells without aspirating any of the medium. On day 14, perform full medium change to mTeSR1 medium and thereafter feed daily until colonies appear (between days 25 and 35). iDot1L treatment can be stopped once small clusters of emerging iPSCs are observed.
10. Once colonies are large enough for manual picking, using a microscope mark the position of the plate where each colony is with a permanent marker. Place a stereo microscope into the laminar flow hood and locate the marked colony. Using a 10 μl

pipette tip, divide the colony into approximately 50 cell clusters and transfer into a VTN-coated well that contains E8 medium. Some of the picked colonies may fail to attach or differentiate therefore we typically pick 12 individual colonies from a reprogramming plate to have additional back-up colonies to expand.

11. After 48 h change the medium and feed daily until the clusters become big enough to be passaged again which will be in 5–7 days. Follow the procedures listed in Section 3.12 for passaging.

3.10 Immunostaining of iPSC Lines

The iPSC lines should express high levels of Oct4 and Nanog which are nuclear proteins and SSEA4, Tra-1-81, and Tra-1-60 which are cell-surface antigens.

1. Place a 12 mm autoclaved cover slip into each well of a 12-well plate using sterile forceps. Add 750 μl PBS/vitronectin solution to each well to coat the surface of the cover slips. Incubate at room temperature for 1 h.
2. Passage one 60–80 % confluent well of iPSC colonies onto the 12-well plate with 1 ml of E8 medium per well. Change the medium everyday After 4–5 days, colonies will be about 2 mm which is proper size for immunostaining.
3. To fix the cells, aspirate the medium and wash once with PBS. Add 500 μl of 4 % PFA in PBS to each well and incubate for 30 min at room temperature. Aspirate PFA and wash three times with PBS (*see* **Note 12**).
4. Permeabilize the cells by adding 500 μl 0.2 % Triton-X solution to each well and incubating for 30 min at room temperature. During the incubation prepare blocking buffer which consists of 3 % BSA and 5 % donkey serum in PBS. Aspirate the Triton-X and wash the cover slips with PBS three times. Add 1 ml blocking buffer into each well and incubate for 2 h at room temperature or overnight at +4 °C. Seal the edges of the plate with parafilm to prevent evaporation.
5. Dilute the primary antibodies at a 1:50–1:200 ratio in blocking buffer (*see* **Note 13**). Place a strip of parafilm in a 10 cm petri plate. Pipet 15 μl of the diluted primary antibody (Oct4, Nanog, Tra-1-81, Tra-1-60, or SSEA4) onto the parafilm. Aspirate the blocking buffer and wash the cover slip with PBS for three times. Using a pair of forceps, lift the cover slips from the 12-well plates and flip them over on top of the droplets containing the primary antibody. Make sure that the cover slips are in the correct orientation such that the cells are in direct contact with the antibody solution.
6. Put a piece of wet paper towel into the 10 cm plate to keep humidified, then seal the cover of the plate with parafilm, and incubate at +4 °C overnight. When using fluorescently labeled antibodies cover the plate with aluminum foil to protect from light.

7. Next day, wash the cover slip three times with PBS and incubate with 1:1000 dilution of the secondary antibody in the dark at +4 °C for 3 h. Wash again for three times and then incubate with a 1:10,000 dilution of DAPI or Hoechst 33342 prepared in 50 % glycerol/PBS for 10 min. Mount the cover slip onto a microscope slide and seal the edges with nail polish. Keep all the slides in the dark until image acquisition using a fluorescence microscope (Fig. 3).

3.11 Testing for Episomal Vector Integration

1. Isolate genomic DNA from one confluent well of iPSCs grown in a 6-well plate using commercial DNA isolation kits as indicated by the manufacturer. Dilute DNA samples to 50 ng/μl. Setup PCR reactions using 50 ng genomic DNA as template per reaction with the following primers: *EBNA-Fwd*:AGGGCCAAGACATAGAGATG, *EBNA-Rev:* GCCAATGCAACTTGGACGTT, *GAPDH-Fwd*: ATCACCATCTTCCAGGAGCGA, *GAPDH-Rev*: TTCTCCATGGTGGTGAAGACG.
2. Untransfected fibroblast DNA can be used as a negative control and any one of the episomal reprogramming plasmid DNA can be used as a positive control. Run the PCR reactions at 95 °C for 3 min, followed by 30 cycles of 95 °C for 10 s, 65 °C for 30 s, and 72 °C for 30 s and a final extension cycle of 72 °C for 5 min.
3. Visualize the PCR products by running them on a 2 % agarose gel (Fig. 4).

3.12 Teratoma Formation

For teratoma formation assays, immunocompromised mouse strains such as SCID or NOD/SCID can be used. All animal experiments must be carried out under protocols approved by the relevant Institutional Animal Care and Use Committees (IACUC).

1. Thaw Matrigel overnight at +4 °C on ice. Prepare an injection solution containing 50 % Matrigel and 50 % cold D10 medium and keep on ice. It is very important to keep Matrigel on ice as it will solidify at room temperature.
2. For each injection at least three confluent wells of a 6-well plate are needed. Detach iPSCs using regular passaging methods (Section 3.9 or 3.12) and collect all the cells in a 15 ml Falcon tube. Centrifuge at 1000 rpm (200 × *g*) for 2 min. Aspirate medium, resuspend pellet in 100 μl cold injection mixture, and keep on ice.
3. Using a 1 ml syringe and 21 G needle, perform subcutaneous or intramuscular injection into properly anesthetized mice. Monitor the mice for 6–8 weeks after injection for teratoma formation at the injection site.
4. Anesthetize the mice and sacrifice via an IACUC-approved method. Carefully dissect out the teratoma, taking care to

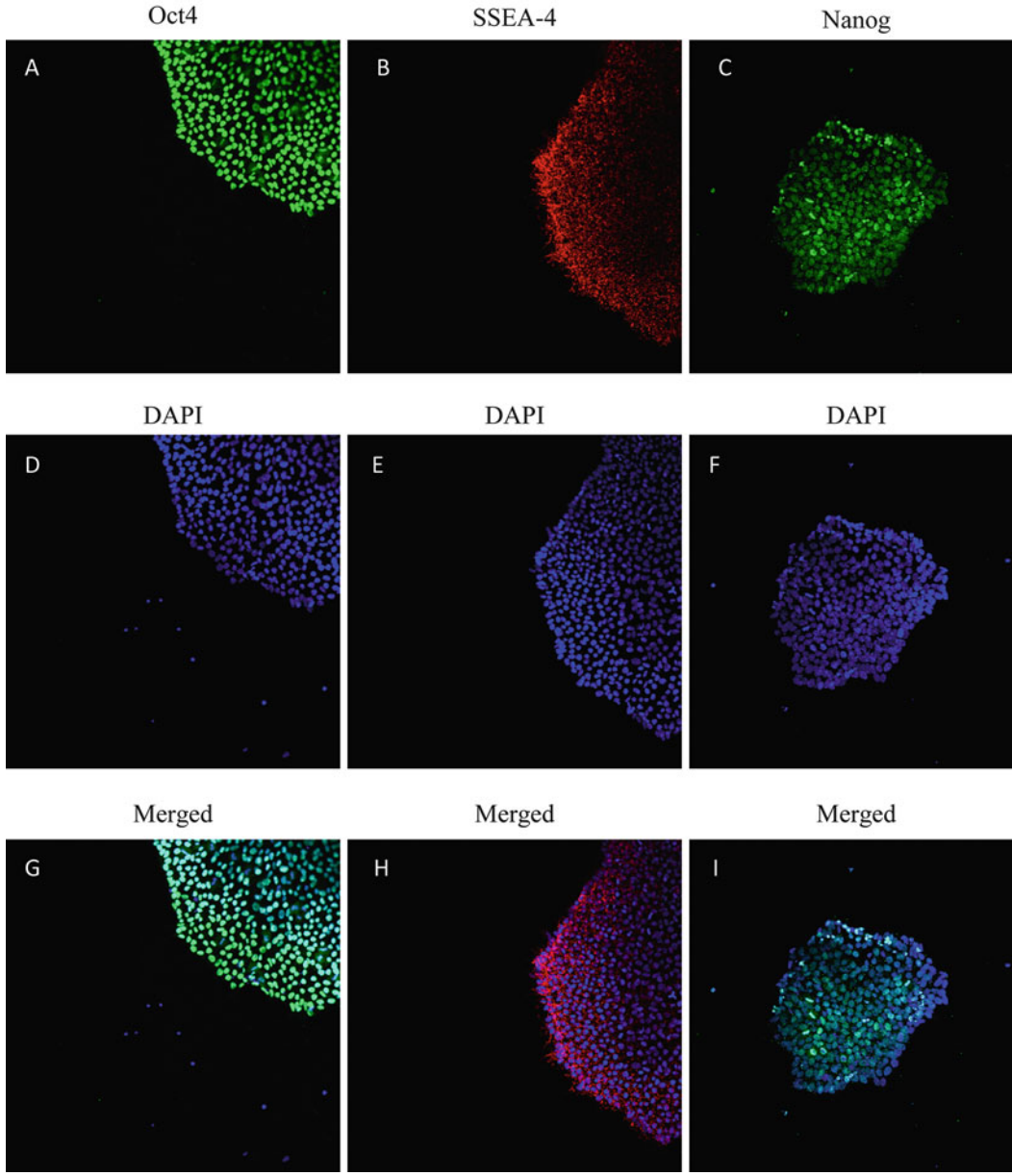

Fig. 3 Immunofluorescence staining of iPSC colonies generated from human PBMCs using episomal vectors. (**a**–**c**) iPSCs immunostained with Oct4, SSEA4, and Nanog antibodies, respectively. (**d**–**f**) DAPI staining. (**g**–**i**) Merged images. All images were taken at 40× magnification

remove it in one piece. Place the tumor mass in a 50 ml Falcon and wash twice with PBS. Fix the tumor by incubating in 10 % formalin for at least 48 h prior to sending it to histopathological staining and examination (*see* **Note 14**).

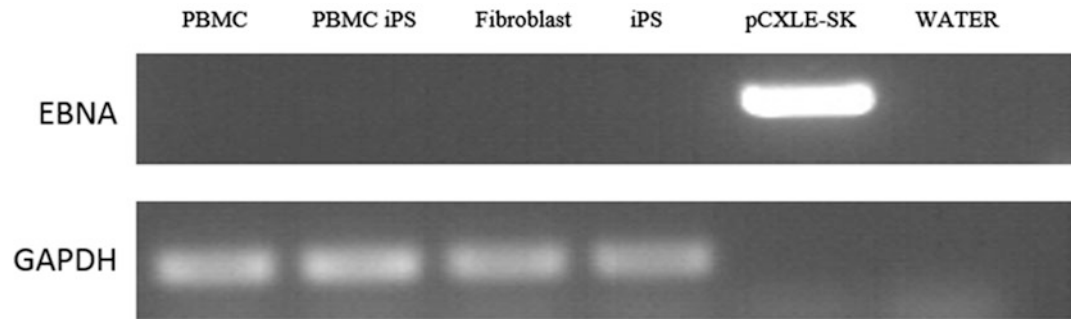

Fig. 4 Testing for genomic integration of episomal vectors in iPSCs by PCR. 1 ng of pCXLE-SK plasmid DNA was used as a positive control. Amplification of GAPDH indicates the presence of genomic DNA in the tested samples

4 Notes

1. Make sure that the plasmids are prepared using an endotoxin-free maxi-prep kit as they will be used in electroporation of patient-derived primary cells. In order to ensure sterility we also filter the final maxi-prep DNA through a 0.22 μm filter.
2. For all cell culture media that contains bFGF, prior to use, warm to room temperature but do not put in a 37 °C water bath. bFGF will lose activity if incubated for long periods of time at 37 °C [13]. This will result in poor reprogramming efficiency and failure to expand iPSC lines.
3. In some cases emergence of fibroblasts may be preceded by the outgrowth of keratinocytes which will grow as epithelial sheets and not as individual cells. If no apparent fibroblast growth is observed after 7 days, biopsy pieces can be kept in culture for up to 3–4 weeks with regular medium changes. During the first trypsinization keratinocytes do not readily come off the plate and fibroblasts are preferentially passaged. Keratinocyte growth is eventually halted and fibroblasts take over the culture in the presence of serum.
4. Fibroblasts can be split at a ratio of 1:3 for up to ten passages. It is advisable to cryopreserve the cells in the first three passages as primary fibroblast cultures will cease proliferation, and become senescent and refractory to reprogramming after ten passages.
5. It is important that the volume of DNAs not exceed 10 % of the total electroporation volume to avoid cell death during the procedure. If the DNAs are too dilute, concentrate by standard ethanol precipitation and adjust the volumes accordingly. It is also important to make sure that the electroporation tip does not contain any air bubbles as this will lead to discharge of an electrical spark and cell death.

6. The optimum settings for maximum transfection efficiency and cell viability is determined experimentally. We generally use a GFP-expressing plasmid to determine the optimal electroporation settings for new cell types. In general, the settings given here for fibroblasts and blood cells will work adequately. For additional cell types, Neon Transfection system has predetermined settings that can be used as starting points.
7. We typically split the cells at a 1:4 to 1:8 ratio into multiple MEF-plated wells. If the fibroblasts become too dense, it becomes difficult to identify and pick the emerging iPSC colonies. Typically, iPSC colonies will start to emerge after 14 days. For some slower growing fibroblast lines, it could take longer up to 30 days. When colonies become large enough to pick, manually passage them onto fresh MEF-coated plates.
8. For one well of 6-well plate use 1 ml VTN solution or 500 μl for one well of a 12-well plate. Shake the plate gently to be sure that VTN covers the entire surface of the wells. Any remaining plates can be sealed with parafilm and stored at +4 °C for up to 2 weeks.
9. When grown on MEFs with hESC medium, iPSC colonies should be passaged as clumps. There should not be any large colonies that can be seen with the naked eyed nor should the colonies be broken down into single cells.
10. The number of cells plated is important to maintain the PBMC in vitro. Therefore, at least 3×10^6 cells should be plated into each well of a 6-well plate. After 6 days of culture, the number of live cells is usually around 1×10^6.
11. The inclusion of the pCXWB-EBNA1 plasmid which expresses the EBNA1 protein has been shown to increase the efficiency of reprogramming of blood cell types. EBNA1 is required for replication of the episomal vectors and is therefore thought to transiently boost the copy number of the reprogramming plasmids and enhance expression of the factors [7].
12. After the fixation step, coverslips can be kept in PBS at +4 °C for 2–3 weeks. For each antibody staining, one cover slip is used. The extra cover slips can be stored as backup.
13. The optimal antibody concentrations have to be determined experimentally but the ratios given here can be used as a starting point. Always include a cover slip which is stained only with the secondary antibodies to detect the background fluorescence.
14. Paraffin-embedded sections can be stained with hematoxylin and eosin or with specific antibodies to determine tissue identity. A trained pathologist will be able to assess whether there are representative cell types from the three germ layers present within the teratomas. Most frequently detected are endodermal glandular structures, neural rosettes, and cartilage.

Acknowledgements

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Generation and Neuronal Differentiation of Patient-Specific Induced Pluripotent Stem Cells Derived from Niemann-Pick Type C1 Fibroblasts

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Abstract

Patient-specific induced pluripotent stem cells (iPSCs) are discussed to provide a powerful tool to investigate pathological mechanisms of diseases. Moreover, such cells might be a future platform for individualized personal treatment of diseases with a broad spectrum of mutations and thus resulting in phenotypical specificities.

Here, we present a protocol for the induction of induced pluripotent stem cells from patient fibroblasts with Niemann-Pick type C1 disease (NPC1). The induction is based on a retroviral system, using the "classical" transcription factors, which were described by Takahashi and colleagues in 2007. To obtain a neuronal in vitro model system of NPC1, human iPSCs were differentiated to neural progenitor cells (NPCs) and subsequently to cells of the neural lineage, namely, neurons and glial cells. iPSCs, NPCs, and terminal neuronal differentiated cells (NDCs) were characterized by means of immunocytochemistry as well as patch clamp recordings and calcium imaging to prove the functional maturation.

Keywords: Retroviral reprogramming, Patient-specific iPSCs, Embryoid bodies, Teratoma, Pluripotency, Immunocytochemistry, Patch clamp, Calcium imaging

1 Introduction

Patient-specific induced pluripotent stem cells were generated by means of a retroviral transduction system mainly based on the protocol used by Takahashi and coworkers (1), providing a reliable, well-described approach with a relatively high efficiency in comparison to insertion free systems. Here, we successfully reprogrammed human dermal fibroblasts by using SOX2, KLF4, OCT4, and c-MYC resulting in induced pluripotent stem cells. The pluripotency was proven by the expression of several markers and the pluripotent differentiation potential in vitro and in vivo. The iPSCs were subsequently differentiated into neural progenitor cells. The neural progenitor identity of these cells was demonstrated by the verification of markers like nestin, Pax6, and Sox2. Finally, NPCs were differentiated into glial cells and functional neurons. Cell types were analyzed by immunocytochemistry, and

functional properties were determined by whole cell patch-clamp recordings and calcium imaging experiments.

Niemann-Pick type C1 (NPC1) disease belongs to the family of lysosomal storage disorders and is based on the mutation of the NPC1 gen, encoding a transmembrane protein. The impaired lipid transport and sequestration result in accumulation of, e.g., cholesterol and gangliosides like GM2 and GM3 in the late endosome and lysosome (2). The clinical manifestation is diverse and shows intrafamilial variability, starting with neonatal icterus and hepatosplenomegaly in early childhood, cerebellar ataxia, seizures, gelastic cataplexy, and vertical supranuclear palsy in adolescence to progressive neurological degradation, psychoses, and dementia in adulthood (3).

The pathogenic mechanisms ultimately leading to a massive degeneration and loss of neurons in the CNS, especially Purkinje cells in the cerebellum, are not exactly understood. Most of our knowledge regarding NPC1 is based on cell models like human fibroblasts (4–6) and animal models like mouse (7), cat (8), and fruit fly (9). The first model system based on human induced pluripotent stem cells was described recently, reflecting the main characteristic phenotype, namely, the accumulation of cholesterol (10).

2 Materials

2.1 General Required Equipment and Materials

As a general rule, the cell culture procedures must be followed using sterile techniques. All media/reagents must be warmed to 37 °C immediately before use.

All staining procedures can be done under non-sterile conditions at room temperature:

1. Humidified incubator at 37 °C and 5 % CO_2.
2. Biosafety and laminar flow cabinet (optional with an integrated binocular).
3. Inverted microscope with a range of phase contrast objectives (×4, ×10, and ×20), fluorescent attachments and optionally equipped with digital camera to take pictures for quality recordings.
4. Tabletop centrifuge (coolable).
5. Liquid nitrogen storage tank.
6. Fridge and freezer (−20 °C, −80 °C).
7. Freezing container filled with isopropanol.
8. Gas burner (small size).
9. Cell counter device, e.g., CASY Cell Counter (Innovatis) or hemocytometer.

10. Electrical or mechanical pipette device and corresponding pipettes.
11. Pipettes and tips.
12. Glass pipettes.
13. Tweezers.
14. Magnetic stirrer.
15. DMEM/F12 (1:1, Life Technologies).
16. DMEM (+ GlutaMAX, Life Technologies).
17. Fetal calf serum (FCS).
18. Penicillin 10,000 U/ml/streptomycin 10,000 µg/ml (Pen/Strep).
19. PBS −/− (without calcium and without magnesium).
20. PBS +/+ (with calcium and with magnesium).
21. DMSO.
22. 15/50 ml tubes (conical).
23. 1.5 ml tubes.
24. Large bore tips for P1000 pipettes (Mettler Toledo).
25. Cryovials.
26. Advanced 6 well plates (Greiner).
27. 4 well plates, e.g., for cover slips (Nunc).
28. Glass cover slips.
29. Paraformaldehyde solution 4 %.

2.2 Generation of Retrovirus and Titer Assay

2.2.1 Cells

1. HEK293FT cells (Invitrogen).

2.2.2 Materials

1. Plasmids (Addgene): c-MYC (18119), KLF4 (17227), OCT4 (17225), SOX2 (17226), gag/pol (14887), VSV-G (8454).
2. X-tremeGENE 9 (Roche).
3. Protamine sulfate (Sigma): prepare a 500 µg/ml stock solution in distilled water.
4. Trypsin/EDTA 0.05 %.
5. 0.45 µm filter unit.
6. 10 cm culture dishes.
7. 12 well plates.
8. Sodium azide.

2.2.3 Medium

1. Basal medium I: DMEM, 10 % FCS.

2.2.4 Solution

1. Analysis buffer: PBS −/−, 0.5 % BSA, 0.02 % sodium azide.

2.3 Generation and Maintenance of iPSCs on Feeder Cells

2.3.1 Cells

1. Human fibroblasts.
2. Feeder cells (inactivated mouse embryonic fibroblasts, Amsbio).

2.3.2 Materials

1. Trypsin/EDTA 0.25 %.
2. Gelatin from porcine skin: prepare a 0.1 % solution, filter it through a 0.22 μm filter unit and store it at 4 °C up to 4 weeks.
3. Valproic acid (Sigma): prepare a 500 mM stock solution in distilled water.
4. ROCK inhibitor Y-27632 (Miltenyi Biotec): prepare a 10 mM stock solution in PBS −/−.
5. Knockout DMEM (Gibco).
6. Knockout serum replacement (Invitrogen).
7. Minimum essential medium nonessential amino acids (MEM NEAA, Gibco).
8. GlutaMAX 100× (Gibco).
9. 2-Mercaptoethanol 1,000× (Gibco).
10. Fibroblast growth factor 2, human (FGF2, Amsbio).
11. Defined FCS (Invitrogen).
12. 3 cm culture dishes.
13. 0.22 μm filter unit.

2.3.3 Media

1. Basal medium I: DMEM, 10 % FCS.
2. Basal medium II: DMEM, 10 % FCS, 1 % Pen/Strep.
3. iPSC medium: DMEM/F12, 20 % knockout serum replacement, 1× MEM NEAA, 1× GlutaMAX, 2× 2-Mercaptoethanol, 0.25 % Pen/Strep, 12,5 ng/ml FGF2.
4. iPSC freezing medium: mix iPSC medium 1:1 with DMSO/defined FCS-mixture (1:4).

2.3.4 Gelatin Coating

1. Prepare a 0.1 % gelatin solution in distilled water.
2. Sterile filter the gelatin solution through a 0.22 μm-filter unit.
3. Cover a 6 well with 1 ml gelatin solution and a cover slip with 500 μl, respectively.
4. Incubate for at least 30 min in the incubator and remove solution before plating feeder cells.

2.4 Verification of Pluripotency Markers

2.4.1 Materials

1. Methanol.
2. Sodium chloride: prepare a 1 M solution in distilled water.
3. TRIS: prepare a 1 M solution in distilled water with pH 9.8.
4. Magnesium chloride: prepare a 1 M solution in distilled water.
5. NBT/BCIP stock solution (Roche).
6. Sodium azide: prepare a 0.02 % solution in distilled water.
7. TRIS hydrochloride.
8. Tween20.
9. Normal goat serum (Dako).
10. Triton-X-100.
11. Bovine serum albumin (BSA): prepare a 10 % solution in PBS −/−.
12. Anti-Nanog (rabbit IgG, Stemgent).
13. Anti-Oct4 (rabbit IgG, Stemgent).
14. Anti-SSEA3 (rat IgM, Stemgent).
15. Anti-SSEA4 (mouse IgG, Stemgent).
16. Anti-Tra-1-60 (mouse IgM, Stemgent).
17. Anti-Tra-1-81 (mouse IgM, Stemgent).
18. Alexa Fluor 488 (goat-anti-mouse IgG, Molecular Probes).
19. Alexa Fluor 488 (goat-anti-mouse IgM, Molecular Probes).
20. Alexa Fluor 488 (goat-anti-rat IgM, Molecular Probes).
21. Alexa Fluor 568 (goat-anti-rabbit IgG, Molecular Probes).
22. DAPI: prepare a 5 mg/ml stock solution in PBS −/−.
23. Glycerol.
24. MOWIOL 4-88 (Sigma).
25. TRIS hypochlorite: prepare a 0.2 M solution in distilled water with pH 8.5.
26. DABCO (Sigma).

2.4.2 Solutions

1. AP staining solution: 75 % distilled water, 10 % 1 M sodium chloride solution, 10 % 1 M TRIS solution, 5 % 1 M magnesium chloride solution, 1:50 NBT/BCIP stock solution.
2. Rinse buffer: PBS −/−, 20 mM TRIS hydrochloride, 0.15 M sodium chloride, 0.05 % Tween 20, pH 8.5.
3. Blocking solution: PBS −/−, 10 % normal goat serum, 0.1 % Triton-X-100, 1 % BSA.
4. First antibody solution: PBS −/−, 0.1 % Triton-X-100, 1 % BSA.

5. DAPI solution: 250 ng/ml DAPI in PBS −/−.
6. Mounting medium: mix 6 g glycerol and 2.4 g MOWIOL 4-88 for 1 h on a magnetic stirrer. Add 6 ml of distilled water and mix for additional 1 h. Add 12 ml of 0.2 M TRIS hypochlorite solution and incubate for 2 h at 50 °C in a water bath. Mix every 20 min on a magnetic stirrer (the components will not be dissolved completely). Spin tube 15 min at 5,000 × *g* to pellet the undissolved components. Dissolve 25 mg DABCO per ml supernatant and mix well. Aliquot the mounting medium and store at −20 °C.

2.5 Evidence of Pluripotent Differentiation Potential In Vitro and In Vivo

2.5.1 Animals

1. Immunodeficient SCID hairless mice (Crl:SHO-*Prkdc*^{scid} Hr^{hr}, Charles River).

2.5.2 Materials

1. Ultra-low adherent 6 well plate (Stemcell Technologies).
2. Knockout DMEM (Gibco).
3. GlutaMAX 100× (Gibco).
4. 2-Mercaptoethanol 1,000× (Gibco).
5. Sodium azide: prepare a 0.02 % solution in distilled water.
6. Anti-nestin (mouse IgG, R&D).
7. Anti-muscle actin (mouse IgG, Dako).
8. Anti-alpha fetoprotein (mouse IgG, Sigma).
9. Trypsin/EDTA 0.05 %.
10. Matrigel (Corning).
11. Syringe 1 ml (Braun).
12. Needle 27 G (Braun).
13. Preparation instruments.
14. Formalin.
15. Ethanol.
16. Paraffin.
17. Xylene.
18. Mayer's hematoxylin solution (Medite).
19. Eosin solution (J. T. Baker).
20. Neo-Mount (anhydrous, Merck).
21. Object covers.

2.5.3 Medium

1. EB medium: knockout DMEM, 20 % FCS, 1× MEM NEAA, 1× GlutaMAX, 1× 2-mercaptoethanol, 0.25 % Pen/Strep.

2.5.4 Equipment

1. Paraffin embedding tool.
2. Microtome.
3. Drying closet.

2.6 Generation and Maintenance of NPCs

2.6.1 Materials

1. Neurobasal medium (Life Technologies).
2. GlutaMAX 100× (Gibco).
3. N-2 supplement (100×) liquid (Life Technologies).
4. B-27 serum-free supplement (50×) (Life Technologies).
5. Human serum albumin (HSA, Albunorm).
6. Fibroblast growth factor 2, human (FGF2, Amsbio): prepare a 10 mg/ml stock solution in PBS −/− in the presence of 0.1 % HSA, aliquot, and store at −20 °C.
7. Epidermal growth factor (EGF, Peprotech): prepare a 10 mg/ml stock solution in 10 mM glacial acetic acid solution (CH_3COOH) + 0.1 % HSA, aliquot, and store at −20 °C.
8. Recombinant mouse Noggin Fc Chimera (R&D): prepare a 500 µg/ml stock solution in PBS −/−, aliquot, and store at −20 °C.
9. SB-431542 (Sigma): prepare a 10 µM stock solution in DMSO, aliquot, and store at −20 °C.
10. Stemgent Stemolecule Y-27632 (Miltenyi biotech): prepare a 10 mM stock solution in PBS −/−, aliquot, and store at −20 °C.
11. Trypsin/EDTA 0.05 %.
12. Benzonase 250 U/µl.
13. Trypsin inhibitor.
14. Tryp/Benzonase: prepare 250 U/ml Benzonase in Trypsin/EDTA, aliquot, and store at −20 °C.
15. Trit/Benzonase: prepare 0.55 mg/ml trypsin inhibitor in DMEM/F12 + 25 U/ml Benzonase, aliquot, and store at −20 °C.
16. HBSS.
17. Poly-L-ornithine (PLO, Sigma): prepare a 15 mg/ml stock solution in PBS −/−, sterile filter it through a 0.22 µm-filter unit, aliquot, and store at −20 °C.
18. Laminin 1 mg/ml (mouse, Amsbio).
19. Glacial acetic acid: prepare a 10 mM solution in distilled water.

2.6.2 Media

1. NIM medium (medium for neural induction): DMEM/F12/neurobasal medium ratio 1:1; 1× GlutaMAX, 1× N2 supplement, 1× B27 serum-free supplement, 0.25 % Pen/Strep, FGF2 5 ng/ml, recombinant mouse noggin Fc Chimera 500 ng/ml, SB-431542 20 μM. To prepare 25 ml of NIM media, combine 11.5 ml DMEM/F12, 11.5 ml neurobasal medium, 0.25 ml GlutaMAX, 0.25 ml N2 supplement, 0.5 ml B27 serum-free supplement, 62.5 μl Pen/Strep, 100 μl recombinant mouse noggin Fc Chimera 500 ng/ml, and 50 μl SB-431542 10 mM. Filter-sterilize, store at 4 °C, and use within 2 weeks. Noggin, SB-431542, and FGF2 should be added prior to use.
2. NPC medium (medium for NPC expansion/differentiation): DMEM/F12/neurobasal medium ratio 1:1; 1× GlutaMAX 1× N2 supplement, 1× B27 serum-free supplement, 0.25 % Pen/Strep (=NPC medium − GF for differentiation). For NPC expansion, add growth factors FGF2 10 ng/ml and EGF 10 ng/ml (=NPC medium + GF). To prepare 250 ml of NPC medium + GF, combine 112 ml DMEM/F12, 112 ml neurobasal medium, 2.5 ml GlutaMAX supplement, 2.5 ml N2 supplement, 5 ml B27 serum-free supplement, 625 μl Pen/Strep, 250 μl FGF2 10 mg/ml, and 250 of EGF 10 mg/ml. Filter-sterilize, store at 4 °C, and use within 2 weeks. FGF2 and EGF should be added prior to use.
3. NPC freezing medium (medium for NPC freezing): 10 % DMSO in NPC medium + GF. Prepare always fresh.

2.6.3 PLO/Laminin Coating

1. Prepare a 15 μg/ml PLO solution in PBS −/−.
2. Use 0.5 ml to coat a cover slip or 1 ml to coat a 6 well.
3. Incubate for 1 h at room temperature.
4. Wash wells with PBS −/− three times.
5. Dilute laminin to 10 μg/ml in ice-cold DMEM/F12.
6. Aspirate PBS −/−, cover wells with laminin solution (0.5 ml/cover slip, 1 ml per 6 well), and incubate for at least 1 h at 37 °C in incubator. Before adding cells, wash the wells with warm DMEM/F12 or store the plates at 4 °C not longer than 1 week.

2.7 Detection of Expressed Neural and Neuronal Markers

2.7.1 Materials

1. Anti-nestin (mouse IgG, R&D).
2. Anti-Sox2 (rabbit IgG, Abcam).
3. Anti-beta III Tubulin (mouse IgG, Santa Cruz).
4. Anti-MAP2 (rabbit IgG, Millipore).
5. Alexa Fluor 488 (goat-anti-mouse IgG, Molecular Probes).
6. Alexa Fluor 568 (goat-anti-rabbit IgG, Molecular Probes).
7. DAPI: prepare a 5 mg/ml stock solution in PBS −/−.

2.7.2 Solutions

1. Blocking solution: PBS +/+, 0.3 % Triton-X-100, 5 % normal goat serum.
2. First antibody solution: PBS +/+, 1 % normal goat serum.
3. DAPI solution: 250 ng/ml DAPI in PBS −/−.
4. Mounting medium: *see* Section 2.4.2.

2.8 Patch-Clamp Recordings and Calcium Imaging Experiments

2.8.1 Materials

1. Potassium chloride (KCl).
2. HEPES.
3. EGTA.
4. Magnesium chloride hexahydrate ($MgCl_2 \times 6H_2O$).
5. Calcium chloride dihydrate ($CaCl_2 \times 2H_2O$).
6. Calcium chloride monohydrate ($CaCl_2 \times H_2O$).
7. Sodium chloride (NaCl).
8. Glucose monohydrate (Glucose $\times H_2O$).
9. Fura2-AM (cell permeant calcium indicator, Life Technologies).

2.8.2 Solutions

1. ICS (intracellular solution): 140 mM KCl, 10 mM HEPES, 11 mM EGTA, 1 mM $MgCl_2 \times 6H_2O$, 1 mM $CaCl_2 \times 2H_2O$, dissolved in distilled water. pH adjusted to 7.2.
2. ECS (extracellular solution): 125 mM NaCl, 2.4 mM KCl, 2 mM $CaCl_2 \times H_2O$, 1 mM $MgCl_2 \times 6H_2O$, 10 mM HEPES, 10 mM glucose $\times H_2O$, dissolved in distilled water. pH adjusted to 7.4.
3. ECS with a high potassium chloride concentration: 125 mM NaCl, 30 mM KCl, 2 mM $CaCl_2 \times H_2O$, 1 mM $MgCl_2 \times 6H_2O$, 10 mM HEPES, 10 mM glucose $\times H_2O$, dissolved in distilled water. pH adjusted to 7.4.
4. Prepare a 5 mM stock solution of Fura2-AM in DMSO.
5. Loading solution for calcium imaging with Fura2-AM: dilute Fura2-AM stock solution to 5 μM loading solution in ECS.

2.8.3 Equipment

1. Standard patch-clamp rig.
2. Motorized upright Zeiss Axioskop2 FS.
3. Motorized manipulator (Luigs + Neumann).
4. EPC10/double patch-clamp amplifier with the Pulse/PulseFit software package (HEKA).
5. Universal puller (Zeitz).
6. Borosilicate glass (GB150F-10, Harvard Apparatus).

7. pH meter.
8. Focal drug application system like ALA OctaFlow (ALA Scientific Products).

2.9 Detection of Cholesterol Accumulations as a Hallmark for NPC1

2.9.1 Materials

1. Filipin from *Streptomyces filipinensis* (Polyscience): prepare a 50 mg/ml stock solution in DMSO, aliquot, and store at −80 °C.
2. SDS: prepare a 0.1 % solution in distilled water.
3. Liquid nitrogen.
4. Pierce BCA Protein Assay Kit (Pierce).
5. Amplex Red Cholesterol Assay Kit (Life Technologies).

2.9.2 Solutions

1. Filipin staining solution: dissolve filipin stock solution (50 mg/ml) to 10 mg/ml in DMSO and afterwards to 0.1 mg/ml in PBS −/−. Prepare always fresh before use.
2. Mounting medium: *see* Section 2.4.2.

3 Methods

3.1 Generation of Retrovirus and Titer Assay

To reprogram human fibroblasts into iPSCs, the generation of retroviral particles encoding for SOX2, KLF4, OCT4, and c-MYC is necessary as well as subsequently determination of the infection efficiency by means of a titer assay.

3.1.1 Generation of Retroviruses

1. For each type of virus seed 3 Mio HEK293FT cells per 10 cm culture dish into ten 10 cm culture dishes in 10 ml basal medium I and let cells attach for 24 h in the incubator.
2. The next day, warm X-tremeGENE 9 to room temperature and mix well. For each 10 cm culture dish, blend 300 µl of DMEM with 20 µl X-tremeGENE 9 in a 1.5 ml tube and incubate for 5 min at room temperature (*see* **Note 1**).
3. Add to each mixture of DMEM/X-tremeGENE 9 2.5 µg DNA of just one viral vector (SOX2, KLF4, OCT4, or c-MYC) plus 0.25 µg DNA of VSV-G and 2.25 µg DNA of gag/pol. Incubate for 20 min at room temperature.
4. Add one DMEM/X-tremeGENE 9/DNA-mixture to one 10 cm culture dish in a dropwise manner and incubate the plates for 18 h in the incubator.
5. After 18 h, remove media and add 7.5 ml fresh basal medium I. Incubate for additional 30 h.
6. Collect the virus containing media and filter it through a 0.45 µm filter unit.

7. (Optional) Ultracentrifuge the virus containing media at 70,000 × *g* and 4 °C for 90 min and solve the pellet in an appropriate volume of DMEM (100 times concentrated is recommended).
8. Aliquot the virus containing media and store at −80 °C.

3.1.2 Titer Assay

For successful reprogramming of fibroblasts, different infections efficiencies of the four retroviruses are essential. To determine the infection rate of the generated viruses, the internal GFP sequence of the viruses is used in a titer assay.

1. Seed 100,000 HEK293FT cells per 12 well in 1 ml basal medium I supplemented with 5 μg/ml protamine sulfate.
2. Add different volumes of one virus to individual 12 wells, e.g., 100 μl, 250 μl, 500 μl, 750 μl, and 1 ml or (if the virus is concentrated) 5 μl, 10 μl, 20 μl, 35 μl, and 50 μl (*see* **Note 2**).
3. Incubate the cells for 48 h.
4. Aspirate the medium, wash the cells with 1 ml PBS +/+, and detach them using 0.5 ml/12 well 0.05 % trypsin/EDTA for 3 min. Stop the enzymatic reaction by adding 1 ml basal medium I and transfer the cell suspension into a 1.5 ml tube.
5. Centrifuge the cell suspension in the tubes for 5 min at 300 × *g*, remove the supernatant, and resuspend the pellet in 100 μl PBS −/−.
6. Fix the cells by adding 100 μl of 4 % paraformaldehyde solution and incubate for 15 min at room temperature.
7. Centrifuge again likewise before and resuspend the pellet in analysis buffer.
8. Perform flow cytometry measuring the green fluorescence of the transfected cells. Use the non-transfected cells as a reference.
9. Determine the virus volume of SOX2, KLF4, and OCT4 for a 70–80 % and of c-MYC for 40–50 % infection efficiency (given by the proportion of green fluorescent cells), respectively.
10. If the efficiency is not in the tested volume range, repeat the experiment with other volumes of the viruses.

3.2 Generation and Maintenance of iPSCs on Feeder Cells

The generation of iPSCs from human fibroblasts needs stable conditions and an uninterrupted work flow; otherwise spontaneous differentiation may occur. Therefore, all steps regarding generation and cultivation should be done within half an hour to avoid cooling down of the culture media.

3.2.1 Generation and Passaging of iPSCs

1. Seed 100,000 human fibroblasts (*see* **Note 3**) into a 3 cm culture dish in 3 ml basal medium I and incubate overnight in the incubator.

2. Aspirate the media and add 3 ml basal medium I supplemented with 5 μg/ml protamine sulfate and the four viruses. Volumes of the viruses SOX2, KLF4, and OCT4 should have shown an infection efficiency of 70–80 % and 40–50 % for c-MYC, respectively.
3. Incubate the cells for 48 h.
4. Wash the cells two times with 2 ml PBS +/+, detach the cells by adding 0.5 ml 0.25 % trypsin/EDTA, and incubate them for 5 min in the incubator. Stop the enzymatic reaction adding 1 ml basal medium II, transfer the cell suspension into a 15 ml tube, and centrifuge it for 5 min at 300 × *g*. Aspirate the supernatant, resuspend the cell pellet in 7.5 ml basal medium II, and plate the cells on a gelatin-coated 6 cm dish. Incubate it for 24 h.
5. Change the medium to iPSC medium supplemented with 0.5 mM valproic acid. Replace medium daily for 1 week.
6. Replace iPSC medium still daily omitting valproic acid. Within the next 25 days, iPSC colonies appear. Microscope the plate every day and observe the morphology changes of the cells (*see* Fig. 1).

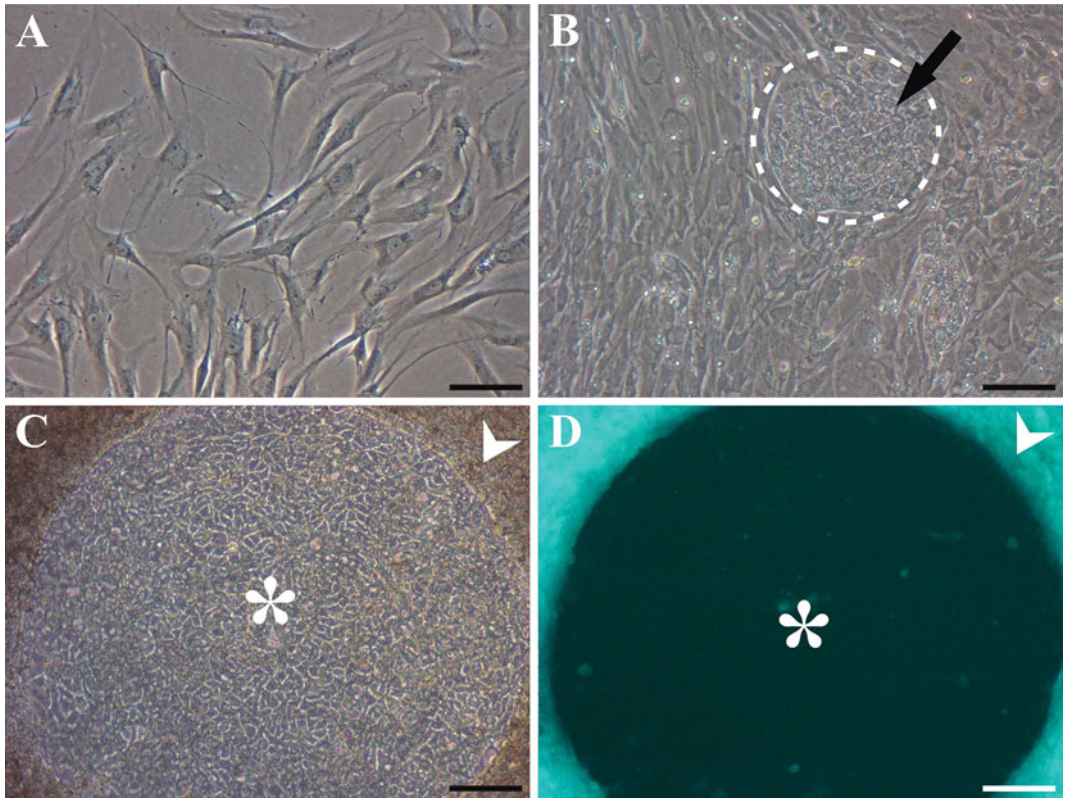

Fig. 1 Fibroblasts, which are seeded for reprogramming (**a**), change their morphology to small and early iPSC colonies (**b**, *white circled area with black arrow*). (**c**) Fully reprogrammed iPSC colonies (*asterisk*) show a round to oval morphology and loose GFP expression (**d**, *asterisk*), while surrounding and not completely reprogrammed fibroblasts (**c**, *arrow head*) still express GFP (**d**, *arrow head*). Scale bar 100 μm

7. At least 24 h before the first picking of colonies, seed inactivated mouse embryonic fibroblasts (feeder cells) into gelatin-coated 4 wells. Therefore, thaw one vial of feeder cells in the 37 °C warm water bath for 2–3 min. Add the cell suspension to 9 ml basal medium II in a dropwise manner. Centrifuge the cells at 300 × *g* for 5 min to remove the DMSO-containing freezing medium. Aspirate the supernatant and resolve the pellet in 10 ml basal medium II. Count the cells and seed 37,500 cells/cm^2. Change the media every third day. Use the cells for a maximum of 10 days.
8. Prepare glass hooks for the picking of iPSC colonies by pulling glass pipettes in the flame of a small gas burner.
9. Aspirate the medium from the feeder cells, wash them twice with DMEM/F12, and add fresh iPSC medium supplemented with 10 μM ROCK inhibitor Y-27632 to prime the feeder cells at least 1 h before picking the iPSC colonies.
10. Change the medium to iPSC medium supplemented with 10 μM ROCK inhibitor Y-27632.
11. For passaging, lift iPSC colonies from the surrounding fibroblasts using the glass hook. If the colony is big enough (diameter ≥500 μm), divide it into two pieces.
12. Transfer one colony or the pieces of one colony to one 4 well with feeder cells using a large bore tip. Gently press the colony onto the feeder cells using the glass hook (*see* **Note 4**).
13. Change the iPSC medium on a daily basis.
14. After 5–8 days, the cells are ready for the next passaging. Follow steps 7–13—if colonies become bigger (diameter ≥1 mm), separate them into more than two pieces (*see* **Note 5**).

3.2.2 Cryopreservation of iPSCs

1. At the day of passaging, divide the colonies into bigger pieces than you would do for passaging. Use iPSC colonies of one 6 well for freezing.
2. Transfer the iPSC containing medium to a 15 ml tube using a large bore tip and centrifuge at 20 × *g* for 1 min.
3. Aspirate the supernatant and add 1 ml of iPSC freezing medium in a drop wise manner while gentle swirling the tube.
4. Transfer the iPSC colonies containing freezing medium into a cryovial using a large bore tip. Store cryovial in a freezing container prefilled with 250 ml room-temperate isopropanol and freeze overnight at −80 °C.
5. Transfer the frozen cryovial to liquid nitrogen for long-term storage.

3.2.3 Thawing of iPSCs

1. Prepare feeder cells at least 24 h before thawing iPSCs (*see* Section 3.2.1, **step** 7).
2. Remove the cryovial from liquid nitrogen and thaw it in 37 °C warmed water bath.
3. Prepare a 15 ml tube with 9 ml iPSC medium.
4. Transfer the thawed iPSC colony pieces from the cryovial to the 15 ml tube in a dropwise manner using a large bore tip. Swirl it carefully.
5. Centrifuge the cells at 150 × *g* for 3 min to remove the DMSO-containing freezing medium.
6. Aspirate the supernatant and resuspend the iPSC colony pieces in 1.5 ml iPSC medium supplemented with 10 μM ROCK inhibitor Y-27632 using a large bore tip.
7. Aspirate the medium from the feeder cells, wash them twice with 1 ml DMEM/F12, and transfer the iPSC medium containing colonies onto it using a large bore tip.
8. Put the plate in the incubator and distribute the iPSC colonies evenly by fast forward and backward movements.
9. Change the medium on a daily basis.
10. After 5–8 days, colonies are ready to be passaged.

3.3 Verification of Pluripotency Markers

In order to verify the pluripotent status of the generated iPSCs, the expression of typical stem cell markers has to be analyzed.

3.3.1 Alkaline Phosphatase Staining

1. Cultivate iPSCs on feeder cells for 5–7 days in a 6 well.
2. Remove the medium and wash cells twice with 2 ml/6 well PBS −/−.
3. Fix the iPSC colonies with 1 ml/6 well ice-cold methanol for 10 min at −20 °C.
4. Remove methanol, wash again with PBS -/-, and incubate the cells with 1 ml/6 well staining solution for 10–15 min at room temperature in the dark.
5. Stop the colorimetric reaction by removing the staining solution and wash with distilled water.
6. Cover the stained iPSC colonies with distilled water. Every pluripotent stem cell colony displays a deep purple color based on high alkaline phosphatase activity. The surrounding feeder cells remain unstained, due to absent alkaline phosphatase activity in these terminal differentiated cells.

3.3.2 Detection of Several Expressed Pluripotency Markers

1. Cultivate iPSCs on feeder cells on gelatin-coated cover slips in 4 well plates for 5–7 days.
2. Aspirate the medium, wash the cells twice by adding 500 μl/cover slip PBS −/−, and fix the cells for 15 min at room temperature by adding 400 μl/cover slip 4 % paraformaldehyde solution.

3. Aspirate the paraformaldehyde solution, wash again with 500 µl/cover slip PBS −/−, and cover the cells with 500 µl/cover slip 0.02 % sodium azide solution until immunocytochemistry will be performed. Up to that point, store the cells at 4 °C.
4. Remove sodium azide solution and wash cells twice with 500 µl/cover slip rinse buffer.
5. Block and permeabilize the cells for 45 min at room temperature by adding 400 µl/cover slip blocking solution.
6. Wash the cells three times with 500 µl/cover slip rinse buffer.
7. Incubate 300 µl/cover slip primary antibody (e.g., Nanog, Oct4, SSEA3, SSEA4, Tra-1-60, and Tra-1-81) diluted 1:100 in antibody solution over night at 4 °C.
8. Remove primary antibody and wash three times with 500 µl/cover slip rinse buffer.
9. Incubate the cells with 300 µl/cover slip secondary antibody diluted 1:500 in PBS −/− for 1 h at room temperature in the dark.
10. Remove secondary antibody and wash three times with 500 µl/cover slip rinse buffer.
11. Incubate the cells with 400 µl/cover slip DAPI solution for 5 min at room temperature in the dark.
12. Remove DAPI solution and wash three times with 500 µl/cover slip rinse buffer.
13. Clean object slides and add one drop of mounting medium.
14. Aspirate rinse buffer and transfer the cover slip out of the 4 well onto the object slide using tweezers.
15. Store the slides at room temperature in the dark until mounting medium is hardened. Afterwards store the slides at 4 °C in the dark until documentation.

3.4 Evidence of Pluripotent Differentiation Potential In Vitro and In Vivo

One major advance of iPSCs is their potential to differentiate into each cell of the three germ layers. In order to proof this feature of the generated iPSCs, one can analyze the random differentiation into cells of all three germ layers by embryoid body and teratoma formation, displaying an in vitro and in vivo assay, respectively.

3.4.1 In Vitro Differentiation by Embryoid Body (EB) Formation

1. On the day of passaging, lift up whole iPSC colonies of one 6 well from the feeder cell layer using a glass hook. Avoid separating them into smaller pieces (*see* **Note 6**).
2. Transfer the colonies containing medium to a 15 ml tube and allow them to settle down.

3. Remove the supernatant and resuspend the colonies in 4 ml EB medium.
4. Transfer the colonies containing EB medium to one 6 well of an ultra-low adherent plate and incubate them for 3 days. During this period, colonies will form spheres so-called embryoid bodies. The stage and environment of a blastocyst are simulated to induce a random differentiation into cells of all three germ layers.
5. Change the EB medium by transfer of the EBs containing medium to a 15 ml tube. Wait until the EBs settle down, remove the supernatant, and add 4 ml of fresh EB medium. Transfer the EBs containing medium back to the 6 well of the ultra-low adherent plate and incubate for 2 more days.
6. Change the EB medium as described and seed 2–3 EBs per gelatin-coated cover slip in 500 µl EB medium. Within 3 days, EBs attach and cells will spread out.
7. Change the media after 3 and 6 days of differentiation.
8. After 9 days of differentiation, fix the cells. Therefore, remove medium, wash with 500 µl/cover slip PBS −/−, and incubate with 400 µl/cover slip 4 % paraformaldehyde solution for 15 min at room temperature.
9. Remove paraformaldehyde solution, wash again with PBS −/−, and cover the cells with 0.02 % sodium azide solution until immunocytochemistry will be performed.
10. Immunocytochemistry is performed against one marker of every germ layer. For example, one can use nestin as a marker for ectoderm, muscle actin as a marker for mesoderm, and alpha fetoprotein as a marker for endoderm. Follow the protocol, which is described in Section 3.3.2, **steps** 2–15.

3.4.2 In Vivo Differentiation by Teratoma Formation

1. On the day of passaging, lift up iPSC colonies of three 6 wells from the feeder cell layer using a glass hook.
2. Transfer the medium containing colonies into a 15 ml tube and centrifuge for 2 min at 200 × *g*.
3. Aspirate the supernatant, resuspend the colony pieces in 1 ml 0.05 % trypsin/EDTA for cell singling, and incubate for 1 min in the water bath at 37 °C.
4. Stop the enzymatic reaction by adding 2 ml basal medium I and centrifuge as described above.
5. Aspirate the supernatant, wash the iPSCs with 1 ml DMEM/F12, and centrifuge again.
6. Aspirate the supernatant and resuspend the iPSCs in 140 µl ice-cold DMEM/F12 and store them on ice.

7. Directly before injection, add 60 μl of Matrigel to the 140 μl of cell suspension. Mix well.
8. Fill a 1 ml syringe with the matrigel-cell suspension directly over a 27G needle.
9. Inject the matrigel-cell suspension subcutaneously in the right flank of the mouse.
10. Teratoma formation should take place within 6–12 weeks. Tumors are visible as solid subcutaneously structures.
11. Sacrifice the mouse when the tumor has a size of approximately 1 cm (or after 12 weeks without tumor growing).
12. Fix the prepared tumor in formalin for 12 h at room temperature. Divide it into two or three smaller pieces (*see* **Note 7**).
13. Dehydrate the tissue using an ascending order of ethanol for 1 h each.
14. Incubate the tissue three times in absolute ethanol for 1.5 h.
15. Incubate the tissue two times in xylene for 1 h.
16. Coat the tissue with paraffin for 1 h.
17. Embed the tissues in a paraffin block and let it harden at 4 °C.
18. Cut the tissue into 4 μm sections using a microtome.
19. Stretch the sections in a 37 °C warm water bath and transfer them on objective slides.
20. Let them dry at 60 °C over night and store them afterwards at room temperature until staining the tissue with hematoxylin and eosin to distinguish different tissue raised from different germ layers (e.g., pigmented epithelia from ectoderm, cartilage from mesoderm, and gut epithelia from endoderm).
21. Wash the slides twice in xylene for 5 min to remove the paraffin.
22. Hydrate the slides in a descending order of ethanol and wash twice with distilled water for 5 min each.
23. Incubate slides in Mayer's hematoxylin solution for 30 s.
24. Blue the tissue by washing with tap water three times for 5 min.
25. Incubate the slides with eosin solution for 1 min to visualize basic structures.
26. Wash the slides twice in distilled water.
27. Dehydrate the tissue using an ascending order of ethanol for 5 min each.
28. Embed the sections with anhydrous Neo-Mount medium and object covers.
29. Take pictures with a microscope and choose tissue of different origin (*see* Fig. 2).

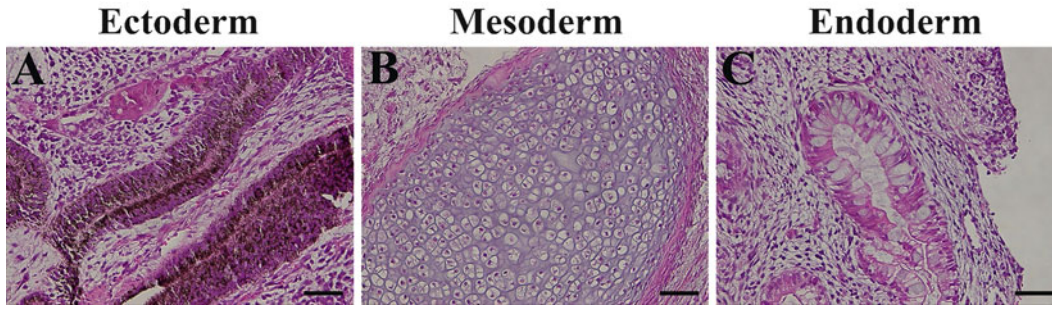

Fig. 2 Hematoxylin and eosin staining of teratoma sections. Teratoma tissue shows structures of different origin like ectodermal pigmented epithelium (**a**), mesodermal cartilage (**b**), and endodermal gut epithelium (**c**). Scale bar 50 μm

3.5 Generation and Maintenance of NPCs

For the differentiation of iPSCs into NPCs, a slightly modified procedure was used, based on a protocol for dual inhibition of SMAD signaling (11).

3.5.1 Generation of NPCs

1. On day 1, remove iPSC medium from exponentially growing, mechanically passaged iPSCs on feeder cells and wash cells twice with 2 ml DMEM/F12 per 6 well.
2. Change medium to 2 ml NIM +10 μM Y-27632 per 6 well.
3. Gently cut large undifferentiated colonies (*see* Fig. 3a) in pieces, leave small colonies whole, and manually detach them carefully with fire-polished hooked glass needle.
4. Transfer the medium containing pieces at a 1:1 ratio (all cells from one 6 well onto another 6 well) in culture dishes coated with PLO/laminin using a large bore tip.
5. Renew medium every day for 9 days with 2 ml/6 well NIM medium without Y-27632 (*see* Fig. 3b).
6. On day 9, wash cells briefly with DMEM/F12 (1 ml per 6 well) and change medium to NPC medium + GF. This step stabilizes the cells as "neural progenitor cells." Renew medium daily.
7. On day 14, use a phase contrast microscope to manually isolate neural rosette structures containing 3D neural tubelike structures present in the center of the colonies (*see* Fig. 3c) by means of fire-polished hooked glass needle. Avoid strictly the outer regions. The center contains the structures of interest and can be easily removed because they have a central lumen, which is not attached to the well.
8. Gently transfer medium containing rosette clusters into a 15 ml tube using a large bore tip. It is important to maintain the NPCs as clusters until trypsinizing for increased cell viability. Place the tube in a 37 °C water bath and allow clusters to settle down.

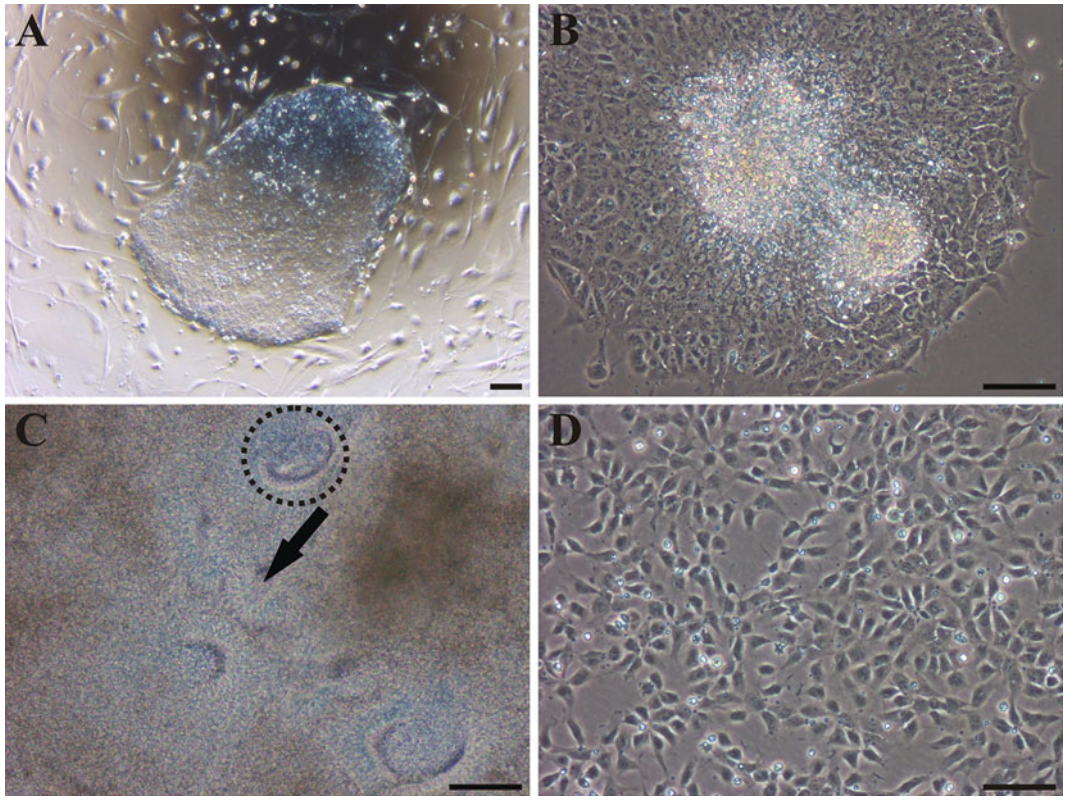

Fig. 3 Undifferentiated iPSC colony on feeder cells (**a**) and one piece of an iPSC colony plated on PLO/laminin in NIM with starting formation of neural rosettes (**b**). (**c**) Neural rosette structures (one in the *black circled area*) with neural tubes in their center (*arrow*) arise and can be isolated. Generated NDCs can be cultured as a monolayer cell line (**d**). Scale bar 100 μm

9. Aspirate supernatant, add 1 ml HBSS, and gently pipette up and down using a large bore tip. Place the tube in a 37 °C water bath and allow aggregates to sink.
10. Remove HBSS and shortly resuspend aggregates in 1,000 μl Tryp/Benz.
11. Incubate 5 min at 37 °C in the water bath.
12. Gently triturate cells and stop reaction by adding 2 ml Trit/Benz.
13. Centrifuge 3 min at 300 × *g* and room temperature.
14. Aspirate supernatant and resuspend cells in 2 ml NPC medium + GF.
15. Perform cell count.
16. Seed 400,000 cells/cm^2 (=Passage 1) in PLO/laminin-coated 6 well (2 ml NPC medium + GF volume) and change medium daily (*see* **Note 8** and Fig. 3d).

3.5.2 Passaging of NPCs

1. Always maintain cells at high densities for optimal growth. Cells are split twice a week, 1 day after they have reached full confluence. Seed 125,000 cells/cm^2 to grow cells for 3 days and 75,000 cells/cm^2 for 4 days, respectively. Cell density at time of passaging is ~350,000–450,000 cells/cm^2.
2. Remove medium and wash cells twice with 2 ml HBSS/6 well.
3. Add 500 µl Tryp/Benz per 6 well and incubate for 2 till 4 min at 37 °C (detach cells by gently tapping). Avoid prolonged incubation time.
4. Stop enzymatic reaction by adding 1 ml Trit/Benz.
5. Transfer cells into a 15 ml tube and centrifuge for 3 min at room temperature and 300 × *g*.
6. Aspirate supernatant and resuspend cells in 1 ml NPC medium + GF.
7. Perform cell count.

3.5.3 Differentiation of NPCs

1. Harvest and resuspend cells in NPC medium—GF to perform a cell count (*see* Section 3.5.2).
2. Plate cells in NPC medium—GF at a density of 50,000–100,000 cells/cm^2 in 500 µl NPC medium—GF on PLO/laminin-coated cover slips and in 1.5 ml NPC medium—GF in PLO/laminin-coated 6 wells, respectively.
3. Carefully renew medium every 3–4 days (500 µl per cover slip and 1.5 ml per 6 well). Within a few days, initial neurite outgrowth is clearly visible, and cells will form a neuronal network within 2 weeks.

3.5.4 Cryopreservation of NPCs

1. On the day of splitting, pellet 2 Mio cells in 1.5 ml tubes at 300 × *g* for 3 min at room temperature.
2. Gently resuspend cells in 1 ml NPC freezing medium in a dropwise manner under gentle swirling.
3. Transfer cells into a cryovial.
4. Place cryovial in a freezing container prefilled with 250 ml room-temperate isopropanol and freeze overnight at −80 °C.
5. Transfer cryovial to liquid nitrogen for long-term storage.

3.5.5 Thawing of NPCs

1. Thaw vial rapidly in 37 °C water bath until one small ice clump is visible.
2. Gently mix cells using a large bore tip, minimize trituration to a minimum, and transfer cells to a 50 ml tube.
3. Add 5 ml NPC medium—GF in a dropwise manner and under gentle swirling and then add slowly 5 ml NPC medium—GF.
4. Pellet cells by centrifugation for 3 min at room temperature and 300 × *g*.

5. Aspirate supernatant and resuspend cells gently in 1 ml NPC medium + GF.
6. Perform cell count.
7. Seed 150,000 cells/cm^2 in a PLO/laminin-coated 6 well and change medium daily (2 ml NPC medium + GF). After 3–4 days, cells are ready to be passaged.

3.6 Detection of Expressed Neural and Neuronal Markers

To identify the neural character of the NDCs, immunocytochemistry against neural progenitor markers like nestin and Sox2 and neuronal markers like beta III Tubulin and MAP2 can be performed.

1. Aspirate the medium and wash the cells on the cover slip by adding 500 µl PBS +/+.
2. Fix the cells for 15 min at room temperature by adding 400 µl/cover slip 4 % paraformaldehyde solution.
3. Aspirate the paraformaldehyde solution and wash cells with 500 µl/cover slip PBS +/+.
4. Permeabilize and block cells with 400 µl blocking solution for 30 min at room temperature.
5. Incubate cells with primary antibody (nestin, Sox2, beta III Tubulin, MAP2) diluted 1:100 in antibody solution for 2 h at room temperature.
6. Aspirate antibody solution and wash cells with PBS +/+ three times for 5 min.
7. Incubate cells with secondary antibodies diluted 1:500 in PBS +/+ for 1 h at room temperature. All following steps should be carried out in the dark to minimize photo bleaching.
8. Remove antibody solution and wash cells with PBS +/+ three times for 5 min.
9. Incubate cells with 400 µl/cover slip DAPI solution for 5 min at room temperature.
10. Remove DAPI solution and wash cells with 500 µl/cover slip PBS +/+ three times for 5 min.
11. Clean object slides and add one drop of mounting medium.
12. Aspirate PBS +/+ and transfer the cover slip out of the 4 well onto the object slide using tweezers.
13. Store the slides at room temperature in the dark for at least 12 h until mounting medium is hardened. Afterwards store the slides at 4 °C in the dark until documentation.

3.7 Patch-Clamp Recordings and Calcium Imaging Experiments

For functional analysis, several standard procedures can be used. The here given procedures are suitable for general functional description and might be adapted (12). Examples of patch-clamp recordings and calcium imaging experiments are shown in Fig. 4.

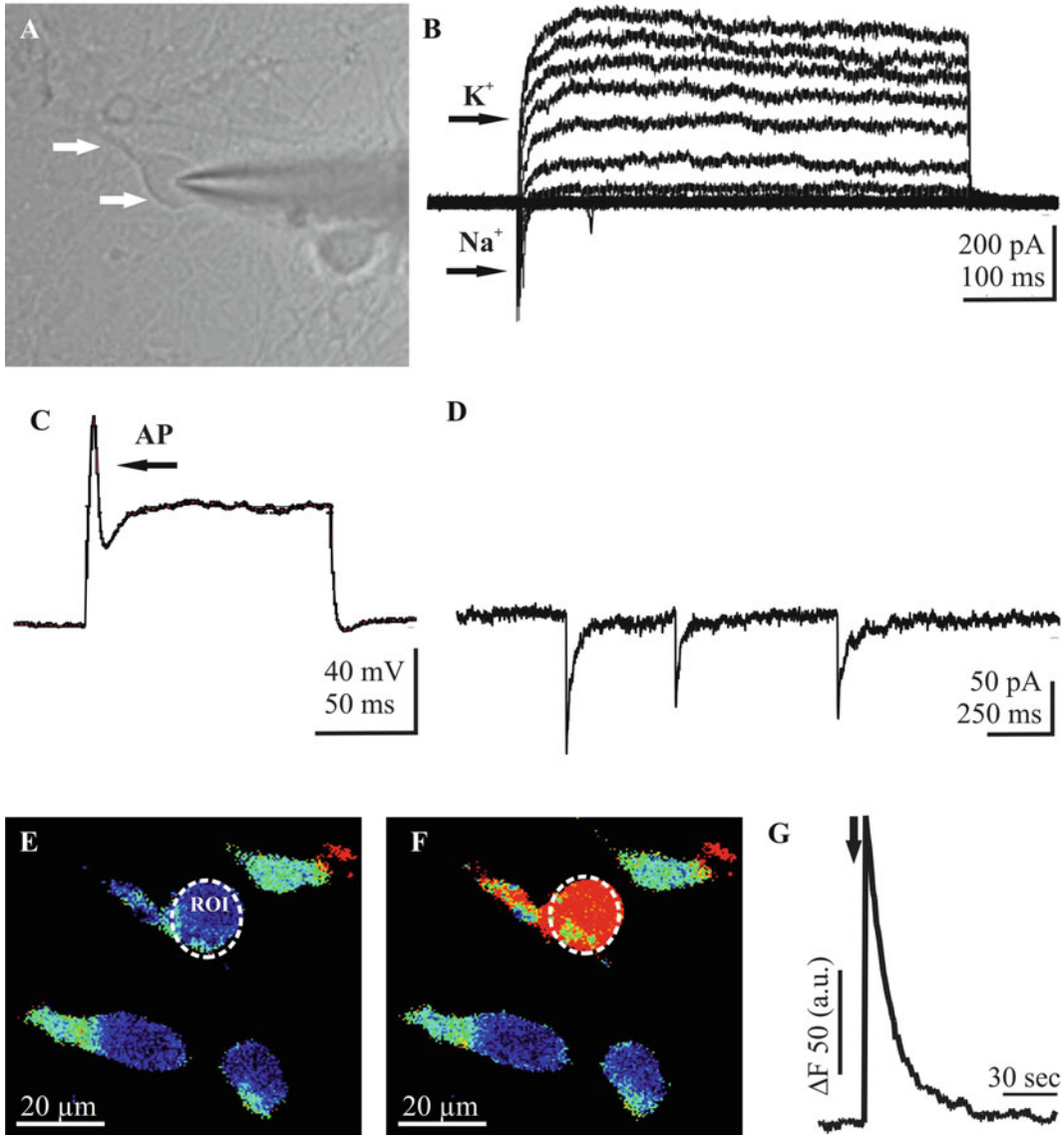

Fig. 4 Example of patch-clamp and calcium imaging data from NDCs. (**a**) NDCs with neuronal morphology were selected under visual control for patch-clamp experiments. *Arrow heads* indicate cell body with a clear process. One can see the patch electrode attached to the cell surface. (**b**) NDCs expressed voltage activated channels like Na_V and K_V, demonstrated by Na^+-inward currents and K^+-outward currents. (**c**) APs can be elicited in the current clamp mode. (**d**) Spontaneous postsynaptic currents, recorded as inward directed currents, demonstrate a functional synaptic transmission between cells. Expression of functional ligand-gated receptors can be proved, e.g., by calcium imaging. NDCs were loaded with the Ca^{2+} indicator Fura2-AM (**e**) and reacted with an increase of the intracellular Ca^{2+} concentration upon the application of the excitatory neurotransmitter glutamate (**f**). (**g**) Time course of a calcium response in differentiated NDCs elicited by the application of glutamate (*arrow* indicates time of application for 1 s)

3.7.1 Patch-Clamp Recordings

1. Cultivate neuronal cells in NPC medium—GF on PLO/laminin-coated cover slips and differentiate until point in time of interest (*see* **Note 9**).
2. Remove culture medium and wash cells with 1 ml ECS.
3. Place cover slip in recording chamber and superfuse cells immediately with ECS.
4. Allow cells to equilibrate for 10–15 min in advance to recordings.
5. Patch electrodes from borosilicate glass pulled with Zeitz Universal puller display resistance of 4–8 MΩ, when filled with ICS.
6. Select cells displaying neuronal morphology, e.g., oval or rectangle cell body with clearly visible processes.
7. After establishing whole cell configuration, clamp cells to a holding potential of −60 or −80 mV in the voltage clamp mode.
8. Compensate series resistance.
9. Use voltage steps families, e.g., with an increment of 10 mV and a pulse duration of 500 ms, to elicit currents mediated by voltage activated ion channels.
10. To record spontaneous postsynaptic currents, clamp cells to a holding potential of −60 or −80 mV and perform a continuous recording in the voltage clamp mode with a gain of 20 mV/pA. Sample data with 10 kHz and filter with 3 kHz using a Bessel filter.
11. Using the above mentioned ECS and ICS, excitatory and inhibitory postsynaptic currents will be visible as inward currents.
12. To elicit currents mediated by ionotropic receptors, e.g., GABA or glutamate receptors, one can use a focal drug application system like ALA OctaFlow.
13. Position barrel of drug application system and use a puff of ECS with a high KCl concentration to check position. Cells should react with an inward current in voltage clamp mode.
14. Clamp cells to a holding potential of −60 or −80 mV and apply short puffs of appropriate agonist.
15. To elicit action potentials switch to current clamp mode and set holding current to zero to obtain the actual membrane potential of the cells.
16. Elicit action potentials by stepwise current injections or record spontaneous action potentials by means of a continuous recording of membrane potential.
17. Examples for above-described measurements are presented in Fig. 4a–d.

18. After the experiment, cover slips can easily be fixed with 4 % paraformaldehyde solution for 15 min at room temperature to perform immunocytochemistry (*see* Section 3.6).

3.7.2 Calcium Imaging

1. Cultivate neuronal cells in NPC medium – GF on PLO/laminin-coated cover slips and differentiate until point in time of interest (*see* **Note 9**).
2. Prepare loading solution and keep it in the dark.
3. Remove culture medium and wash cells two times by adding 500 µl/cover slip ECS.
4. Add 500 µl/cover slip of loading solution and keep cover slip at room temperature for 30 min in the dark.
5. Remove loading solution and wash cells twice by adding 500 µl/cover slip of ECS.
6. Add 500 µl/cover slip of ECS and keep cells at room temperature for 30 min in the dark.
7. Place cover slip in recording chamber and superfuse cells immediately with ECS.
8. Allow cells equilibration to reach stable baseline for 5–10 min in advance to recordings.
9. Basic settings for stable measurement might be given by manufacturer of the imaging system.
10. To record spontaneous calcium transients in, e.g., proliferating cells, one can record signals with 1 Hz.
11. To elicit changes of the internal calcium concentration via calcium permeable ion channels like glutamate receptors, one can use a focal drug application system as described above.
12. Examples for above-described measurements are presented in Fig. 4e–g.
13. After the experiment, cover slips can easily be fixed with 4 % paraformaldehyde solution for 15 min at room temperature to perform immunocytochemistry (*see* Section 3.6).

3.8 Detection of Cholesterol Accumulations as a Hallmark for NPC1

To prove the NPC1 phenotype, cholesterol was visualized by filipin staining and cholesterol accumulation was quantified using the Amplex red assay.

3.8.1 Cellular Visualization of Cholesterol by Filipin Staining

1. Cultivate iPSCs, NPCs, NDCs, or original fibroblasts on cover slips under the cell type-specific conditions.
2. Wash each cover slip with 500 µl PBS +/+.
3. Fix cells for 15 min at room temperature by adding 400 µl/cover slip 4 % paraformaldehyde solution.

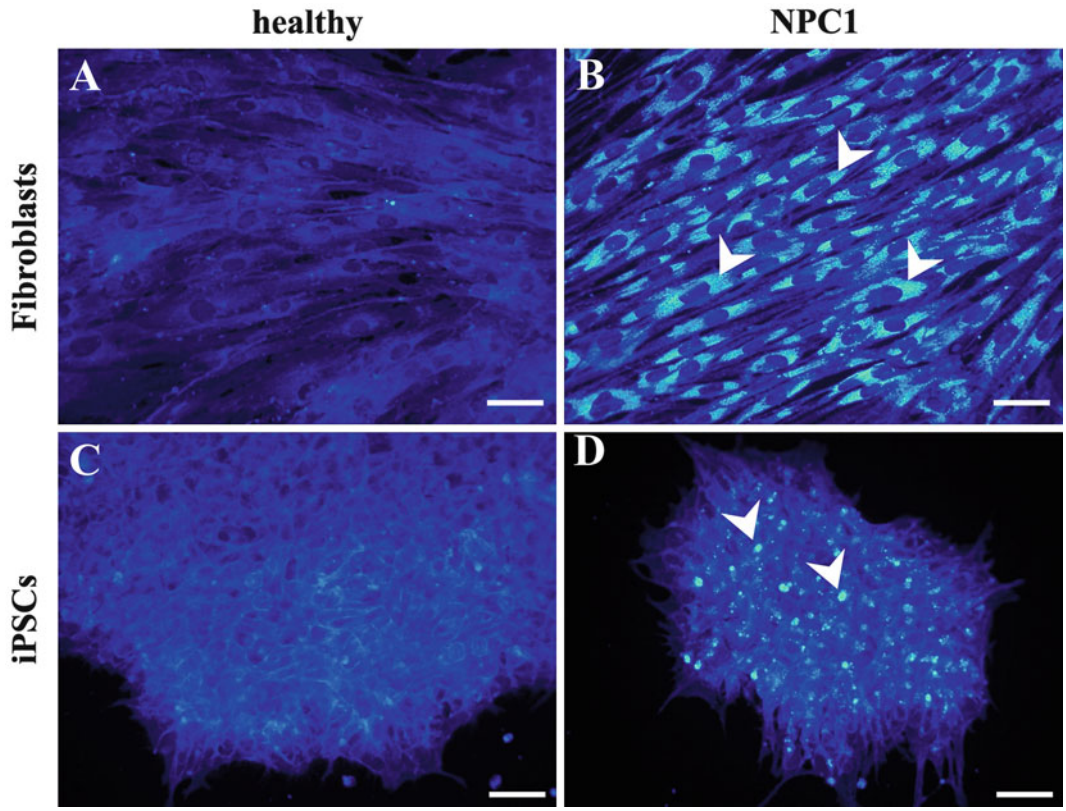

Fig. 5 Filipin staining of fibroblasts (**a**, **b**) and iPSCs (**c**, **d**). Healthy cells (**a**, **c**) show a regular *blue* staining in contrast to the cells with NPC1 (**b**, **d**), which display cholesterol accumulations as *bright areas* (*arrow heads*). Scale bar 50 μm

4. Wash cells twice with 500 μl/cover slip PBS +/+.
5. Incubate cells with filipin staining solution for 45 min in the dark.
6. Wash cells twice with 500 μl/cover slip PBS +/+.
7. Clean object slides and add one drop of mounting medium.
8. Aspirate PBS +/+ and transfer the cover slip out of the 4 well onto the object slide using tweezers.
9. Store slides at room temperature in the dark for at least 12 h until mounting medium is hardened.
10. The next day, take pictures using a microscope with fluorescence unit (*see* **Note 10** and Fig. 5).

3.8.2 Quantification of Cholesterol by Amplex Red Assay

1. Cultivate iPSCs (*see* **Note 11**), NPCs, NDCs, or original fibroblasts in one 6 well under the cell type-specific conditions.
2. Wash and harvest cells as a single-cell suspension.
3. Count cells.

4. Transfer cell suspension in a 1.5 ml tube and centrifuge at 500 × *g* for 5 min.
5. Remove supernatant (cell pellets may be stored at −80 °C until further analysis).
6. Dissolve cell pellets in 100 up to 500 µl 0.1 % SDS solution (*see* **Note 12**).
7. Vortex the solution and shock frost it in liquid nitrogen for 30 s.
8. Thaw solution in tap water for 5 min at room temperature.
9. Repeat **step** 6 and 7 four times to lyse the cells.
10. (*Optional*) If necessary, samples can be stored at −20 °C over night. Thaw them at room temperature to continue the procedure.
11. Determine the protein amount (in mg) by "Pierce BCA Protein Assay Kit" following the manufactures instructions (*see* **Note 13**).
12. Determine the whole cholesterol amount by "Amplex Red Cholesterol Assay Kit" and following the manufactures instructions (*see* **Note 13**).
13. Quantify the cholesterol amount at the rate of cholesterol in µg per mg of protein.

4 Notes

1. X-tremeGENE 9 should not contact the tube wall.
2. Prepare one well as a control without adding any virus.
3. Fibroblasts should not be older than five passages; otherwise chance for reprogramming is lowered.
4. Expand just one colony to a stable iPSC line to avoid the mixture of stable and unstable growing iPSCs. Therefore, use for every new colony a fresh glass hook and change the media between the picking of different colonies.
5. Over the first 10–20 passages, you can expand the cells first for cryopreservation and later on for experiments like immunocytochemical stainings. If you observe iPSC colonies and iPSC lines, respectively, which lose their stem cell like morphology, discard them.
6. For every transfer and medium change, it is recommended to use a large bore tip.
7. This is helpful to see structures in more than one layer than cutting the whole tumor.
8. High plating density is essential for optimal growth since cells need cell-cell contacts, and significant cell death occurs during the first two passages. Within the first 72 h after seeding, many

attached cells will die and remaining cells proliferate. When seeding 400,000 cells/cm^2, plate will be densely confluent after about 3 days.

9. Plating numbers are important as plating cells in high density may result in compact monolayer structure, which can be washed off very easily by the superfusion system. PLO/laminin-coated glass cover slips are easily to handle, obtain good adherence of the cells, and show a low auto-fluorescence in imaging experiments.
10. The filipin staining is not very stable, so take pictures directly 24 h after staining procedure.
11. For this analysis, iPSCs should be cultivated feeder-free on matrigel (13).
12. Volume depends on the cell number. Solution should not be slimy and easy to pipet.
13. Samples can be diluted up to 1:5.

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Multisystemic Disease Modeling of Liver-Derived Protein Folding Disorders Using Induced Pluripotent Stem Cells (iPSCs)

Amy Leung and George J. Murphy

Abstract

Familial transthyretin amyloidosis (ATTR) is an autosomal dominant protein-folding disorder caused by over 100 distinct mutations in the transthyretin (*TTR*) gene. In ATTR, protein secreted from the liver aggregates and forms fibrils in target organs, chiefly the heart and peripheral nervous system, highlighting the need for a model capable of recapitulating the multisystem complexity of this clinically variable disease. Here, we describe detailed methodologies for the directed differentiation of protein folding disease-specific iPSCs into hepatocytes that produce mutant protein, and neural-lineage cells often targeted in disease. Methodologies are also described for the construction of multisystem models and drug screening using iPSCs.

Keywords: Amyloidosis, Disease modeling, Induced pluripotent stem cells, Protein folding disorders, Directed differentiation, Drug screening

1 Introduction

Amyloidosis refers to a group of diseases caused by the extracellular deposition of misfolded fibrillar proteins, leading to multiorgan failure and death (1). Familial amyloidoses (AF) occur when inherited point mutations in the genes coding for abundant serum proteins such as transthyretin (TTR), fibrinogen, lysozyme, or apolipoproteins lead to clinical disease. The most common form of AF arises from aggregation of mutated TTR, a 55 kDA transport protein predominantly synthesized by the liver (1–3). Familial transthyretin amyloidosis (ATTR) is a lethal, autosomal dominant disease caused by single amino acid substitutions arising from one of more than 100 described mutations in the transthyretin gene (2). These single amino acid substitutions destabilize the native homotetrameric structure of circulating TTR, promoting release of amyloidogenic TTR monomers, fibril formation, and deposition as amyloid in target end organs (1–4). Most amyloidogenic TTR variants target the nervous system and heart, inducing neuropathy and cardiomyopathy (2, 5–7). Although transgenic mouse models

and immortalized human cell lines have provided some insights into disease pathogenesis (8–11), these systems are independent of the genetic context of the patient.

The generation of induced pluripotent stem cells (iPSC) through the reprogramming of somatic cells from patients with inherited diseases provides an unprecedented opportunity to study the effects of genetic abnormalities and disease progression. The derivation of unlimited quantities of the relevant cell types targeted and affected in patients with hereditary disease allows for the investigation of the cellular, molecular, and epigenetic events involved in a multisystem, genetic disease such as ATTR. Although most cases of ATTR are due to a single base pair mutation predominantly expressed in the liver, end organ damage occurs outside the liver, highlighting the need for a model capable of recapitulating the multisystem complexity of the disease. iPSC lines can be established that are genetically identical to the individual from whom they are derived, allowing for disease modeling and development of novel therapeutics in the exact genetic context of the patient. Harnessing the pluripotency of iPSC, this protocol illustrates the modeling of multisystemic disease through the directed differentiation of patient-specific iPSC into hepatocytes that produce mutant protein as well as cardiomyocytes and neurons targeted and damaged by deposits of misfolded TTR. Patient-specific, cell-based models such as this facilitate study of the genetic and epigenetic factors in disease, permitting pharmacogenomic assessments of novel therapeutics.

2 Materials

All media and additives are prepared in a laminar flow hood and filter-sterilized using a 0.22 μm filter. All cytokines and growth factors are prepared by reconstituting lyophilized pellets with PBS containing 0.5 % BSA and frozen at −80 °C (*see* **Note 1**).

2.1 iPSC Culture Reagents

1. iPSC Media: mTeSR1 Complete Kit for hESC Maintenance (Stem Cell Technologies 5850). The 5× TeSR1 supplement is thawed at room temperature or overnight at 2–8 °C. We routinely add 100 μg/ml primocin (InvivoGen) to our iPSC media (*see* **Note 2**).
2. hESC Qualified Matrigel: Matrigel (BD 354277) is thawed on ice or at 2–8 °C overnight (*see* **Note 3**).
3. Dispase: (Stem Cell Technologies 07913) Aliquot a 100 ml bottle of stock dispase solution (5 mg/ml) by thawing and dispensing into smaller volumes (10 or 20 ml). Store the aliquots at −20 °C and thaw as desired (avoid repeated freeze–thaw cycles). Prepare a working solution of dispase at 1 mg/ml by diluting 1:5 in DMEM/F-12. Diluted dispase can be stores at 2–8 °C for up to 3 weeks.

2.2 Hepatic Specification Reagents

1. Definitive Endoderm Specification (Days 1–4): STEMdiff Definitive Endoderm Kit (Stem Cell Technologies 05110) (*see* **Note 4**).
2. Hepatic Specification (Days 5–6): Base SFD (Serum-Free Differentiation) Media: 375 ml IMDM, 125 ml Ham's F12, 2.5 ml N2-supplement (Invitrogen 17502-048), 5.0 ml B27-supplement (Invitrogen 12587-010), 3.3 ml of a 7.5 % bovine serum albumin (BSA)/PBS solution, 5 ml of 100 mM L-glutamine (Invitrogen 25030-081), and primocin to a final concentration of 100 μg/ml. Sterile-filter and cover with foil. For Day 5–6 media, add the following to 25 ml of Base SFD: 50 ng/ml activin A, 10 ng/ml BMP4, 10 ng/ml FGF2, 10 ng/ml VEGF, 50 μg/ml ascorbic acid, and 1.5 ml of diluted monothioglycerol (MTG) (13 μl of MTG into 1 ml of IMDM).
3. Hepatic Specification (Days 7–12): Add the following to 25 ml of Base SFD: 50 ng/ml BMP4, 10 ng/ml FGF2, 10 ng/ml VEGF, 10 ng/ml EGF, 20 ng/ml TGFα, 100 ng/ml HGF, 0.1 μM dexamethasone, 50 μg/ml ascorbic acid, and 1.5 ml of diluted monothioglycerol.
4. Hepatic Specification (Days 13–18): Add the following to 25 ml of Base SFD: 10 ng/ml FGF2, 10 ng/ml VEGF, 10 ng/ml EGF, 100 ng/ml HGF, 20 ng/ml oncostatin M, 6 μg/ml vitamin K, 1.5 μM gamma secretase inhibitor (Santa Cruz), 1 % DMSO, 0.1 μM dexamethasone, 50 μg/ml ascorbic acid, and 1.5 ml of diluted monothioglycerol.
5. Hepatic Specification (Days 19–25): Add the following to 25 ml of Base SFD: 100 ng/ml HGF, 20 ng/ml Oncostatin M, 6 μg/ml Vitamin K, 0.1 μM dexamethasone, 50 μg/ml ascorbic acid, and 1.5 ml of diluted monothioglycerol.

2.3 Neuronal Specification Reagents

1. Neural Induction (Days 1–20): STEMdiff Neural Induction Medium (Stem Cell Technologies 05839).
2. Neural Progenitor Expansion (Days 21–28): STEMdiff Neural Progenitor Medium (Stem Cell Technologies 05833).

2.4 Cell Functionality and Viability Assay Reagents

1. Viability Assay Reagents: Hoechst 33342 (Sigma-Aldrich B 2261): Prepare solution at a final concentration of 8.8 ng/ml. Propidium Iodide (Invitrogen): 1,000× stock solution is 1 mg/ml in water.

3 Methods

3.1 Culture of iPSCs in Feeder-Free Conditions

1. Dispensing Matrigel onto Culture Plates: Thaw Matrigel on ice and immediately use to coat tissue culture-treated plates (for a 6-well plate, use 1 ml of Matrigel per well). Swirl the plate to

spread the Matrigel solution evenly across the surface. Coated plates should then be left at room temperature (15–25 °C) for 1 h before use. Do not remove the Matrigel solution until the plate(s) are ready to be used to avoid dehydration. When ready to seed cultures, gently tilt the plate(s) onto one corner and allow the excess Matrigel solution to collect in one corner. Remove the Matrigel solution using a serological pipette or by aspiration. Immediately add mTeSR medium and iPSCs (*see* below).

2. Seeding iPSCs on Matrigel Coated Plates: Warm required amount of mTeSR reagent before adding to culture plates (recommended use is 2.0–2.5 ml of mTeSR per well of a 6-well plate). Seed cells at relatively high density, taking care to maintain cells as aggregates of approximately 50–60 μm in diameter (*see* **Note 5**). Culture the cells at 37 °C, with 5 % CO_2 and 95 % humidity. Full-medium changes must be performed daily with pre-warmed media.
3. Passaging iPSCs Grown in Feeder-Free Conditions: iPSCs are ready to passage when the colonies are large, beginning to merge, and have centers that are dense and phase-bright compared to their edges. Depending on the size and density of seeded aggregates, cultures are usually passaged 5–7 days after seeding. Remove spent medium from iPSC cultures and rinse with PBS (2 ml/well). Add 1 ml dispase and incubate at 37 °C for 7 min (*see* **Note 6**). Remove dispase and gently rinse each well twice with 2 ml of DMEM/F-12 per well to dilute away any remaining dispase. Add 2 ml/well of mTeSR media and scrape cells off with a cell scraper or serological pipette tip. Transfer the detached cell aggregates to a 15 ml conical tube and rinse the well with an additional 2 ml of mTeSR to collect any remaining aggregates. Add the rinse to the 15 ml conical containing cells. Centrifuge the 15 ml tube containing the cells at 300 × *g* for 5 min at room temperature. Remove the supernatant and add 1–2 ml/well mTeSR media. Resuspend pellet gently by pipetting up and down with a P1000 micropipettor (1–2 times). Ensure that the cells remain as aggregates (*see* **Note 5**). Plate the iPSCs with mTeSR media onto a new plate coated with Matrigel. Move the plate in several quick, short, back-and-forth and side-to-side motions to disperse cells across the surface of the wells. Return the plate to the incubator.

3.2 Hepatic Specification of iPSCs in Feeder-Free Conditions

1. Day 0: Passing cells to start the differentiation: Grow cells on Matrigel-coated plates in mTeSR until they are ready to passage. Visually inspect cells and aspirate/remove any differentiated colonies (*see* **Note 7**). Dispase-treat, dislodge colonies as above, and transfer cells to a 15 ml conical for centrifugation. During centrifugation, prepare single cell passaging medium by

adding 1 μg/ml of Rho Kinase Inhibitor (ROCK) to mTeSR which will increase cell viability following the creation of a single cell suspension necessary for efficient differentiation. Following centrifugation, aspirate the supernatant and resuspend the pellet in 1 ml of single cell passaging medium, pipetting up and down 35× with a p1000 pipette. Perform a cell count by adding 10 μl of cells to 10 ml of diluent on a Coulter Counter (set at 7–15 μM) or by counting manually. Replate the cells at a density of 1×10^6 cells/well on a 6-well plate (*see* **Note 8**). If available, place plate(s) in a hypoxic incubator (5 % O_2) and grow cells there for the rest of the differentiation.

2. Day 1 of Hepatic Specification: Aspirate spent media and replace with 2.0 ml of Stem Cell Technologies STEMdiff Definitive Endoderm Kit Medium 1.
3. Days 2–4 of Hepatic Specification: Aspirate spent media and replace with 2.0 ml of Stem Cell Technologies STEMdiff Definitive Endoderm Kit Medium 2.
4. Day 5 of Hepatic Specification: Pass cells to expand to more wells and collect cells for ckit/cxcr4 (endoderm) staining. Prepare fresh Matrigel plates onto which to pass the Day 5 cells. Coat plates with Matrigel for 1 h at room temperature, aspirate Matrigel, wash each well with 2 ml of DMEM/F12, and add Day 5 media + ROCK inhibitor to each well.

   Aspirate spent media, and employ the passaging procedure as noted above with the following caveat: before replating cells, pipette up and down 20 times before dispersing cells into the plates.
5. ckit/cxcr4 Staining for Endoderm on Day 5: For this procedure, collect either a whole well of cells from a 6-well plate or take a portion of a well that you are passing for further differentiation. Resuspend cells in 1 ml of mTeSR media and pipet up and down 40× with a P1000 to get cells in single cell suspension and perform a cell count. Centrifuge the cells at 300 × *g* and 4 °C for 5 min to pellet. Aspirate supernatant and resuspend in 250 μl of PBS+ (PBS supplemented with 0.5 % Fetal Bovine Serum (FBS)). Adjust volume depending on the number of tubes you need per sample (usually one unstained, one isotype, one single antibody stain, and one double antibody stain). For undifferentiated control cells, you only need unstained and double antibody controls. For differentiated cells, you only need one isotype per cell line and one of each of the single antibody stains. For each condition, aliquot 50 μl of cells into 1.5 ml eppendorf tubes on ice. Add the recommended dilution of antibodies or isotype controls (*see* **Note 9**) to each sample, vortex all tubes and incubate on ice for 30 min, covered in foil. Following antibody incubation, top off the tubes with 1 ml of PBS+, vortex, and spin at 300 × *g* and

4 °C for 5 min. Aspirate samples carefully, and resuspend in 300–400 μl of PBS+, pipet up and down 10–15×, and place on ice for FACS analysis (*see* **Note 10**).

6. Day 6 of Hepatic Specification: Aspirate spent media and replace with Day 5–6 media.
7. Days 7–25 of Hepatic Specification: Aspirate spent media and replace with respective media as outlined in Section 2 (*see* **Note 11**). At Day 25 of specification, cells can be collected for transcriptional analyses via quantitative PCR (qPCR), cell surface analyses via FACS, and supernatants can also be collected for examination of secreted proteins via ELISA, immunoblotting, or mass spectrophotometry analysis.

3.3 Neuronal Specification of iPSCs in Feeder-Free Conditions

1. Day 0: Passing cells to start the differentiation: Grow cells on Matrigel-coated plates in mTeSR until they are ready to passage. Visually inspect cells and aspirate/remove any differentiated colonies (*see* **Note 7**). Dispase treat, dislodge colonies as above, and transfer cells to a 15 ml conical for centrifugation. During centrifugation, prepare single cell passaging medium by adding 1 μg/ml of Rho Kinase Inhibitor (ROCK) to mTeSR which will increase cell viability following the creation of a single cell suspension necessary for efficient differentiation. Following centrifugation, aspirate the supernatant and resuspend the pellet in 1 ml of single cell passaging medium, pipetting up and down 35× with a p1000 pipette. Perform a cell count by adding 10 μl of cells to 10 ml of diluent on a Coulter Counter (set at 7–15 μM) or by counting manually. Replate the cells at a density of 1×10^6 cells/well on a 6-well plate (*see* **Note 8**).
2. Day 1–20 of Neuronal Specification: Pre-warm STEMdiff Neural Induction Medium, rinse the wells once with 1 ml of sterile PBS, and add 2 ml STEMdiff Neural Induction Medium per well. Replace spent medium daily with fresh STEMdiff Neural Induction Medium.
3. Day 21–28 of Neuronal Specification: Pre-warm STEMdiff Neural Progenitor Medium, rinse the wells once with 1 ml of sterile PBS, and add 2 ml STEMdiff Neural Progenitor Medium per well. Replace spent medium daily with fresh STEMdiff Neural Progenitor Medium.
4. As cells expand, and depending on the confluency of your cells, it is advisable to split the cells on the following days: 6–9, 12–15, 18–21, and 25–28. Pass the cells as noted above in the hepatic specification protocol.
5. Plating iPSC-derived Neuronal-lineage Cells in Assays (*see* **Note 12**): Dissociate cells as above, and resuspend the single cell suspension in Neural Progenitor Medium with

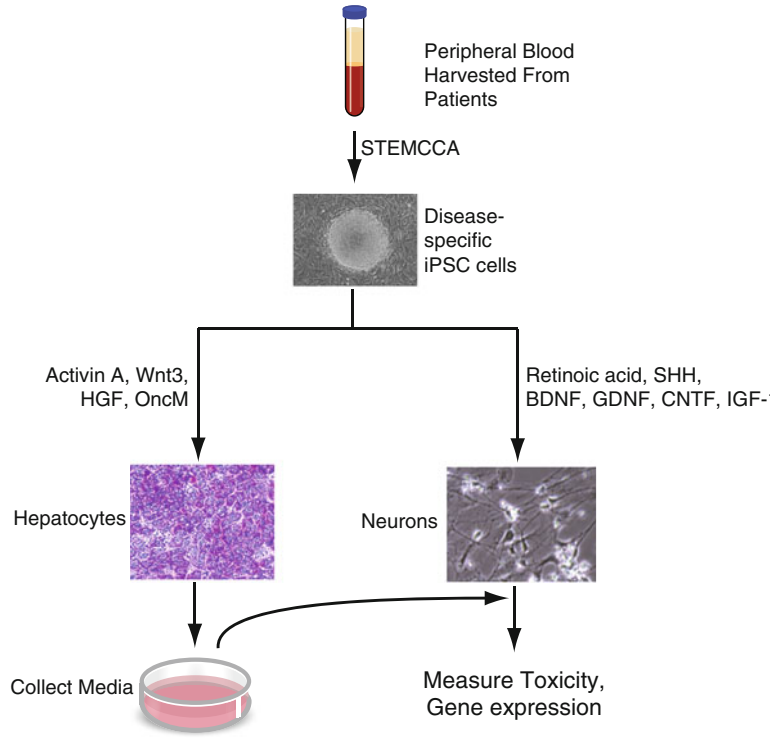

Fig. 1 Illustration showing the iPSC-derived cellular model of hereditary amyloidosis

1 μg/ml laminin and 25 mM beta-mercaptoethanol. Seed dissociated cells at 0.1–0.5 million per well in a lysine/laminin coated 8-well slide, 24-well plate, or 96-well plate. Cryopreserve unused cells using standard freezing medium (~1 million cells per vial). Change Neural Progenitor Medium every other day.

3.4 Model Construction and Cell Viability Assays

The overall design of this flexible, patient-derived cellular model of hereditary amyloidosis is illustrated in Fig. 1.

1. Having successfully derived both the effector hepatic lineages that produce disease-specific proteins and neural-lineage cells, the peripheral target cells in many disease processes, it is then possible to assess the cellular and molecular effects of exposure to aberrant proteins in the target cells. To do this, serum-free conditioned media hepatic supernatants are harvested from control and disease-specific iPSC-derived hepatic cells and exposed to neuronal-lineage target cells for 12–15 days (*see* **Note 13**).
2. Cell Viability Assay: Dissociate cells at room temperature for 3–5 min and wash with PBS+ twice before incubation with 8.8 ng/ml Hoechst 33342 in PBS-FBS buffer at 37 °C for 15 min. Wash cells twice with PBS+ and incubate with 1 μg/ml

propidium iodide in PBS, and immediately analyze by flow cytometry. Cells should be assayed using a BD LSRII flow cytometer (BD Biosciences) or comparable device. Decreased cell survival will be ascertained by a decrease in the proportion of cells in the FSC vs. SSC gate and an increase in propidium iodide positive cells (*see* **Note 14**).

4 Notes

1. Potent cytokines and growth factors are essential for both the maintenance and directed differentiation of pluripotent stem cells. Aliquot reconstituted reagents to avoid multiple freeze–thaw cycles. Once cytokines are added to a formulation, it is not recommended that the working media be used in excess of 2 weeks. We recommend adding cytokines and growth factors to media on each day of use in order to ensure potency. Cytokines are generally sourced from R&D Systems, Minneapolis, MN and are purchased in large quantities to reduce cost.
2. Mycoplasma contamination of cell cultures is widespread, ranging from 5 to 35 % in published reports. There are over 100 species, which is one reason why a single test can fail to detect contamination. Mycoplasma can reach 10^8 cells/ml of tissue culture media without obvious cloudiness and may have no apparent effect on cell growth. Infection undermines all experimental findings as mycoplasmas have been shown to alter virtually every cell culture property and characteristic measured. There is significant financial and personal cost to mycoplasma outbreaks. To reduce the risk of mycoplasma contamination in the CReM, we have instituted a strict policy for the quarantine and testing of new cells, surveillance for mycoplasma contamination within internal cell lines and recommend the routine use of anti-mycoplasma antibiotics (Primocin) in iPSC cell culture of confirmed negative samples. We employ 10 cm plates of antibiotic-free FG293 cells ("surveillance plates") in each incubator with each lab member with cells in culture responsible for the inoculation of the surveillance plate in their incubator with 100 μl of media from each cell line in culture to be used for subsequent testing.
3. Matrigel should be aliquoted into sterile, prechilled 1.5 ml vials. Be sure to keep tubes on ice while dispensing the Matrigel.
4. After several years of using our own formulations for endoderm specification, the CReM now employs the Stem Cell Technologies Definitive Endoderm Kit for the first 4 days of our protocol. In head to head testing with our own formulations, we have found this kit to be extremely reliable while cutting down variability in the process.

5. Culture density is a critical aspect of maintaining iPSCs in an undifferentiated state. Cultures that are either too sparsely or too densely populated can lead to spontaneous differentiation. Initially, seeding multiple wells at various densities may be necessary to determine optimal seeding densities for the iPSC line being used. In addition, preparation of a uniform suspension of suitably sized iPSC clumps for passaging is very important for the successful culture of iPSCs. If he clumps are too large, an increased rate of differentiation within the colonies may occur. If the clumps are too small with many single cells present, cell survival will be compromised.
6. During dispase incubation, colony edges will appear slightly folded back while still adhering to the plate.
7. The efficiency of your differentiations will be directly proportional to your starting culture: if the culture is pristine with very few spontaneously differentiated cells, then your subsequent specifications will work well.
8. The Stem Cell Technologies Protocol says to plate 2×10^6 cells/well for a 6-well plate, but the cells are extremely confluent by Day 5. We have found that plating 1–1.5 $\times$ 10^6 cells/well works very well and even plating as low as 700,000 cells/well still works well.
9. Antibody Information: ckit antibody (BioLegend #313206), ckit isotype (Invitrogen #MG105), cxcr4 antibody (Invitrogen #MHCXCR404), cxcr4 isotype (Invitrogen #MG2a04).
10. At Day 5 of specification, robust co-expression of two markers of definitive endoderm (ckit and cxcr4) is essential for efficient continued differentiation toward the hepatic lineage.
11. Prior to using a new media (i.e., on days 7, 13, and 19), wash wells 1$\times$ with serum-free DMEM/F12 prior to refeeding with fresh media. This will ensure that any growth factors from a previous formulation have been removed.
12. This neural specification protocol is designed to produce large numbers of neural-lineage cells for assays requiring robust populations of cells such as FACS analyses and other functional assays. Importantly, at the completion of this relatively short protocol, although the cells will express many markers of the neuronal lineage, end-stage specification to a distinct neuronal lineage may require the implementation of a separate, additional specification scheme.
13. Hepatic supernatants should be stored at 4 °C and not frozen to ensure the integrity of secreted proteins. The amount of secreted protein in hepatic cultures can be quantified via immunoblotting or mass spectrophotometry.

14. This system is amenable to the testing of small molecule therapeutics which may have efficacy in preventing cellular damage. iPSC-derived neuronal target cells can be dosed with small molecule drugs before the addition of hepatic supernatants to assess their potency in this regard.

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In Vitro Modeling of Alcohol-Induced Liver Injury Using Human-Induced Pluripotent Stem Cells

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Abstract

Alcohol consumption has long been associated with a majority of liver diseases and has been found to influence both fetal and adult liver functions. In spite of being one of the major causes of morbidity and mortality in the world, currently, there are no effective strategies that can prevent or treat alcoholic liver disease (ALD), due to a lack of human-relevant research models. Recent success in generation of functionally active mature hepatocyte-like cells from human-induced pluripotent cells (iPSCs) enables us to better understand the effects of alcohol on liver functions. Here, we describe the method and effect of alcohol exposure on multistage hepatic cell types derived from human iPSCs, in an attempt to recapitulate the early stages of liver tissue injury associated with ALD. We exposed different stages of iPSC-induced hepatic cells to ethanol at a pathophysiological concentration. In addition to stage-specific molecular markers, we measured several key cellular parameters of hepatocyte injury, including apoptosis, proliferation, and lipid accumulation.

Keywords: Induced pluripotent stem cells, Alcoholic liver disease, Hepatic differentiation, Liver steatosis, Apoptosis

1 Introduction

Alcoholic liver disease (ALD) is one of the major causes of morbidity and mortality in the world (1, 2). It includes a broad range of progressive disease stages: fatty liver, liver fibrosis, liver cirrhosis, and hepatocellular carcinoma (3). Approximately 80–90 % of individuals with excessive alcohol consumption develop liver steatosis, of which 20–40 % progress into liver fibrosis without abstinence (2). Heavy alcohol consumption during pregnancy can alter the development of multiple organs in the fetus, including the brain, heart, and liver, leading to fetal alcohol spectrum disorders (FASD) (4). The reported hepatic abnormalities in subjects with FASD include hyperbilirubinemia, elevated levels of liver enzymes, and hepatomegaly, suggesting that excessive alcohol intake can lead to structural liver defects. Liver biopsy from a child with FASD has demonstrated parenchymal fat with portal and perisinusoidal fibrosis, which resembles the changes in adult human ALD (5). Animal studies have also demonstrated a wide range of liver defects under

prenatal alcohol exposure (6, 7). Thus, both animal and human studies provide compelling evidence on alcohol-induced liver injury and dysfunction.

Significant challenges remain for developing preventive or curative approaches targeting ALD (8–11). This is in part due to a lack of human-relevant model systems to study alcohol effect on liver development and regeneration (12). In recent years, human-induced pluripotent stem cells (iPSCs) have been generated from diverse human somatic cells (13–16), which can then be differentiated into a spectrum of mature human cell types including functional hepatocytes (17). This development enables us to access an unlimited supply of hepatocytes, which has been one of the major challenges in the past. Moreover, human iPSCs retain the same genetic information of the donor (i.e., patient) tissues, making iPSCs a promising resource to study human genetic or acquired diseases.

We have established human-induced pluripotent stem cell lines from healthy donors and multiple liver disease patients (13–15, 18). Using our stepwise hepatic differentiation protocol, iPSCs can be induced to definite endoderm (DE), hepatic progenitor cells (HP), and then mature hepatocyte-like cells (MH) under defined conditions (13–15, 17). This in vitro process has been designed to recapitulate human liver development. Here we exposed different stages of iPSC-induced hepatic cells to alcohol (ethanol) at a pathophysiological concentration (100 mM) (19). We observe that exposure to ethanol at the pathophysiological dosage significantly reduces the expression of AFP, an early hepatic cell marker, and induces cell apoptosis, during differentiation of iPSC-derived endoderm into hepatic progenitor cells. Proliferative activity of more mature stage hepatic cells is significantly lowered. Increased amounts of lipid droplets are detected in ethanol-treated iPSC-derived hepatocytes compared to controls.

2 Materials

This study was performed in accordance with the Johns Hopkins Intuitional Stem Cell Research Oversight regulations and followed approved protocols by the Johns Hopkins Institutional Review Board.

2.1 Human-Induced Pluripotent Stem Cell (iPSC) Lines

The human iPSC lines used in this study were previously generated from diverse healthy donor tissues (13–15) and cultured in a feeder-free condition (mTeSR1 medium and Matrigel-coated plates).

2.2 Human iPSC Culture Reagents

1. Matrigel hESC qualified matrix (BD Biosciences, Cat. No. 354277), store at −20 °C.
2. Human iPSC culture medium: mTeSR1 Medium Kit (STEMCELL Technologies, Cat. No. 05850).

3. DMEM/F12 medium (Corning, Cat. No. 10-092-CV), store at 4 °C.
4. Collagenase Type IV (Sigma, Cat. No. C5138-5 g), store at 4 °C. Prepare 1 mg/ml collagenase IV solution with DMEM/F12 and filter for sterilization. Store the collagenase solution at 4 °C.
5. Accutase solution (Sigma, Cat. No. A6964-100 ml), store at −20 °C.
6. Cell scraper 25 cm (Sarstedt, Cat. No. 83.1830).
7. Y-27632 dihydrochloride (a ROCK inhibitor; Tocris Bioscience, Cat. No. 1254-50 mg). Make 20 μl stock aliquots of 100 mM in PBS and store at −20 °C. For use in experiments, thaw the frozen aliquot, dilute the 20 μl in 380 μl PBS to yield a 5 mM solution, and store at 4 °C. The desired final concentration to treat cells is 2–10 μM.
8. Penicillin-streptomycin (Gibco, Cat. No. 15140-122), store at −20 °C.
9. CryoStem Freezing Medium (Stemgent, Cat. No. 01-0013-50), store at 4 °C.
10. Tissue-culture-treated 12-well plastic plates (Thermo Scientific, Cat. No. 130185).

2.3 Hepatic Differentiation Reagents

1. RPMI 1640 Medium with GlutaMAX (Gibco, Cat. No. 61870-036), store at 4 °C.
2. Recombinant human Activin A (R&D systems, Cat. No. 338-AC/CF). Make 25 μl stock aliquots of 1 mg/ml in PBS and store at −80 °C. For use in experiments, thaw the frozen aliquot, dilute the 25 μl in 225 μl RPMI1640 medium to yield a 100 μg/ml solution, and store at 4 °C. The desired concentration to treat cells is 100 ng/ml.
3. B27 supplement (Gibco, Cat. No. 17504-044), aliquot, and store at −20 °C.
4. CHIR 99021 (Tocris, Cat. No. 4423, GSK-3 inhibitor). Make 250 μl stock aliquots of 20 mM in DMSO and store at −20 °C. For use in experiments, thaw the frozen aliquot, dilute the 250 μl in 250 μl DMSO to yield a 10 mM solution, and store at 4 °C. The desired final concentration to treat cells is 1–2 μM.
5. William's Medium E, no glutamine (Gibco, Cat. No. 12551-032), store at 4 °C.
6. Insulin-Transferrin-Selenium (ITS) (Corning Cellgro, Cat. No. 25-800-CR), store at 4 °C. This solution contains 1,000 mg/l human recombinant insulin, 550 mg/l human recombinant transferrin, and 0.67 mg/l selenious acid.
7. HEPES solution (Sigma, Cat. No. H0887-100 ml), store at 4 °C.

8. GlutaMAX Supplement (Gibco, Cat. No. 35050-061), store at 4 °C.
9. Dexamethasone (Sigma, Cat. No. D8893). Make stock aliquots of 10 mM in DMSO and store at 4 °C.
10. Gentamicin solution (Sigma, Cat. No. G1397-10 ml), store at 4 °C.
11. Recombinant human FGF-4 (R&D systems, Cat. No. 235-F4/CF). Make 50 μl stock aliquots of 500 μg/ml in PBS and store at −80 °C. For use in experiments, thaw the frozen aliquot, dilute the 50 μl in 200 μl PBS to yield a 100 μg/ml solution, and store at −20 °C. The desired concentration to treat cells is 10 ng/ml.
12. Recombinant human HGF (R&D systems, Cat. No. 294-HG/CF). Make 50 μl stock aliquots of 500 μg/ml in PBS and store at −80 °C. For use in experiments, thaw the frozen aliquot, dilute the 50 μl in 200 μl PBS to yield a 100 μg/ml solution, and store at −20 °C. The desired concentration to treat cells is 10 ng/ml.
13. Recombinant Human Oncostatin M (OSM) (R&D systems, Cat. No. 295-OM/CF). Make 50 μl stock aliquots of 500 μg/ml in PBS and store at −80 °C. For use in experiments, thaw the frozen aliquot, dilute the 50 μl in 200 μl PBS to yield a 100 μg/ml solution, and store at −20 °C. The desired concentration to treat cells is 10 ng/ml.
14. Fetal bovine serum (HyClone, Cat. No. SH30070.03). Make aliquots and store at −20 °C.
15. Hepatocyte culture medium (HCM): The base HCM contains 15 mM of HEPES, 1 % of GlutaMAX, 1 % of ITS solution, 0.1 μM of dexamethasone, and 0.1 % of gentamicin in William's Medium E. Filter this base medium and store at 4 °C. Prior to use in cell culture, supplement this medium with HGF, FGF-4, and OSM, each at final concentration of 10 ng/ml.

2.4 Antibodies Used in the Study (Table 1)

1. TRIzol Reagent (Life technologies, Cat. No. 10296-028).

2.5 Primer Probes Used for Real-Time PCR (Table 2)

2. High Capacity cDNA Reverse Transcription Kit (Life technologies, Cat. No. 4368813).

2.6 Other Reagents and Kits

3. 0.5 % Oil Red O solution (Sigma, Cat. No. O1391).
4. Annexin V Apoptosis Detection Kit (BD Pharmingen, Cat. No. 559763).

Table 1
Details of antibodies used in this study

Antibodies	Reactive	Source	Supplier	Cat. No.	Dilution
Sox17	Human	Mouse	R&D systems	mab1924	1:200
AFP	Human	Rabbit	Dako	A0008	1:200
ALB-FITC	Human	Rabbit	Dako	F0117	1:200
SSEA3-Fitc	Human	Rat	eBioscience	14-8833-80	1:100 (FACS)
CXCR4-PE	Human	Mouse	eBioscience	12-9999-73	1:100 (FACS)
Ki67	Human	Mouse	Cell Signaling	9449S	1:500
AAT	Human	Goat	Thermo	PA126908	1:100

Table 2
TaqMan primer-probe sets used for real-time PCR

Gene	Abbreviation	Assay ID
Eukaryotic 18S rRNA	18S	Hs03003631-g1
SRY (sex-determining region Y)-box 17	Sox17	Hs00751752_s1
Alpha-fetoprotein	AFP	Hs00173490_m1
Albumin	ALB	Hs00910225_m1
Fatty acid synthase	FASN	Hs01005622_m1

3 Methods

3.1 Matrigel Coating of 12-Well Plates for Pluripotent Stem Cell Culture

The whole procedure should be performed under extra aseptic condition.

1. Thaw frozen Matrigel overnight at 4 °C. Prepare 1:4 dilution of Matrigel using chilled DMEM/F12. Prepare 5 ml aliquots with chilled pipettes and freeze them at −20 °C.
2. Thaw one 5 ml aliquot on ice.
3. Transfer the thawed aliquot to cold 500 ml DMEM/F-12 medium, mix well, and keep on ice.
4. Add 1 ml/well diluted Matrigel into 12-well plates using chilled pipettes. Wrap the coated plates using aluminum foil and incubate at room temperature for 1 h. Store in 4 °C (*see* **Note 1**).
5. When the plate is ready for iPSC culture, bring the plate to room temperature.

6. Remove the Matrigel solution. Ensure that the coated surface is not scratched by pipette.
7. Immediately add 0.5 ml/well iPSC culture medium and then plate cells.

3.2 Human iPSC Culture and Hepatic Differentiation

3.2.1 Human iPSC Culture: Thaw iPSC Lines Onto Matrigel-Coated 12-Well Plates (See **Note 2**)

Culture the cells in mTeSR1 medium at 37 °C with 5 % CO_2. Observe the morphology of the colonies under the microscope and change medium every day. When the colonies are large and ready to merge, passage the cells with Accutase or collagenase IV (*see* **Note 3**).

3.2.2 Differentiation of Human iPSCs to Hepatocytes (Fig. 1)

It is important that the iPSC colonies be evenly distributed and reach 40–80 % confluence before starting differentiation.

1. Day 0: Replace iPSC culture medium with warm RPMI medium supplemented with 100 ng/ml Activin A and 1–2 μM CHIR99021 (*see* **Note 4**). Incubate the cells at 37 °C with 5 % CO_2.
2. Day 1–4/5: Continue replacing the previous culture medium with fresh RPMI supplemented with 100 ng/ml Activin A and 0.05–2 % B27 daily (*see* **Note 5**). After day 4, more than 90 % of the cells express endodermal markers like Sox17 and CXCR4 (Fig. 1b, c).
3. Day 4–9/10: Change medium to RPMI supplemented with 100 ng/ml Activin A, 0.1–1 % B27, 10 ng/ml HGF, and 10 ng/ml FGF-4 every day. Following HGF/FGF-4 treatment, a majority of cells express hepatic progenitor marker AFP (Fig. 1d).
4. Day 9/10–20: Change medium to HCM supplemented with 1–3 % FBS, 10 ng/ml HGF, 10 ng/ml FGF-4, and 10 ng/ml OSM daily (*see* **Note 6**). At day 20, 80–90 % of cells express characteristic mature hepatocyte markers including albumin (ALB) and alpha-1 antitrypsin (AAT) (Fig. 1e, f).

3.3 Alcohol Exposure Method and Effects of Alcohol on Hepatic Differentiation

Alcohol exposure in this model is achieved by directly adding ethanol into multistage hepatic cell culture at a final concentration of 100 mM. Constant alcohol atmosphere was maintained in the culture wells by using a microclimate chamber to prevent alcohol evaporation (20).

1. Effects of alcohol on differentiation of iPSCs into definitive endoderm (DE): Treat the iPSCs with 100 mM ethanol from day 0 to 3 of endoderm differentiation. Prepare the medium containing ethanol freshly every day before use. At day 4, harvest the cells for real-time PCR, flow cytometry, and

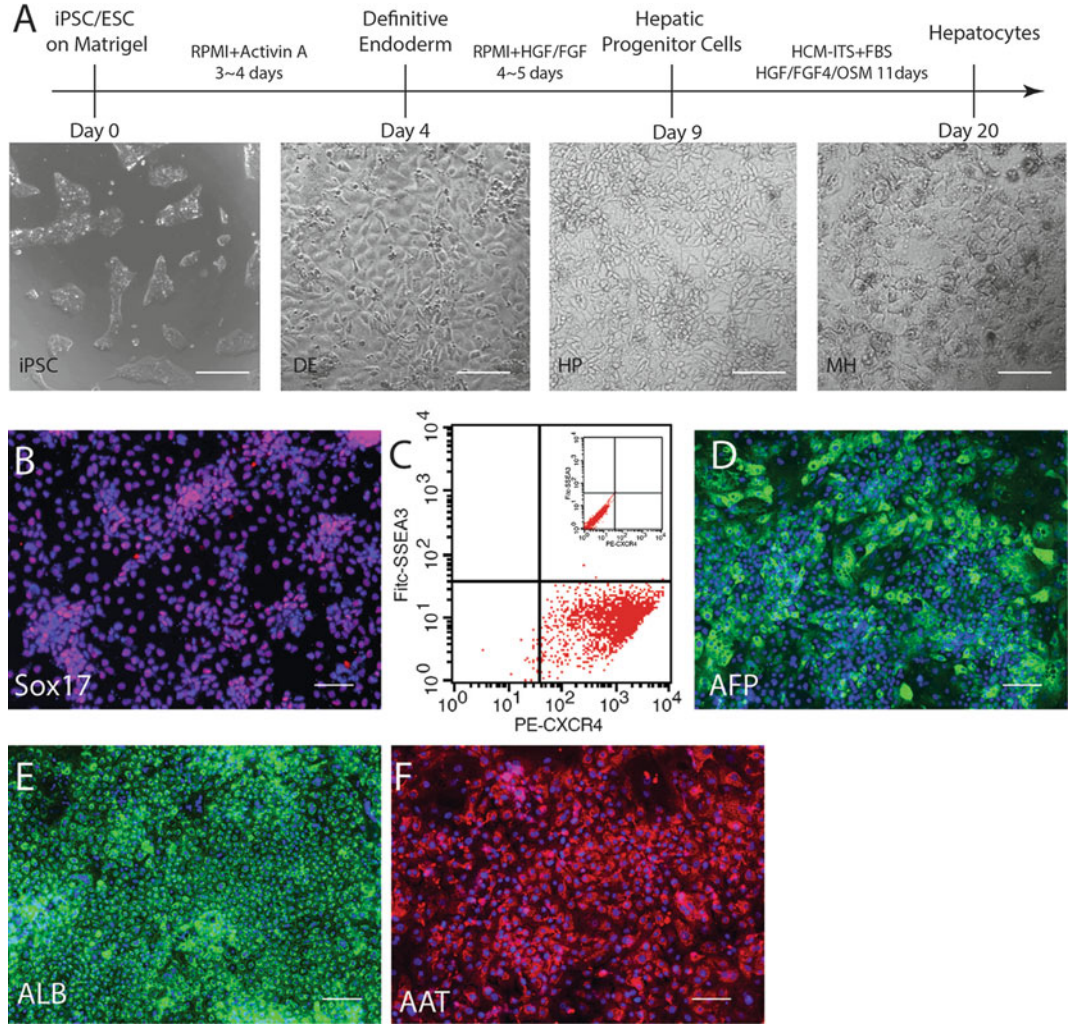

Fig. 1 Differentiation of human iPSCs into multistage hepatic cells. (**a**) A schematic diagram of hepatic differentiation procedure and corresponding bright field images of hepatic cells at each stage. (**b**) A representative image showing the expression of Sox17 (*red*), a definitive endoderm marker, at differentiation day 4. (**c**) Flow cytometric analysis shows that ~99 % of cells express CXCR4, another definitive endoderm marker, at day 4. (**d**) Expression of AFP (*green*), a hepatocyte progenitor marker, at day 9 after initiation of hepatic differentiation. (**e** and **f**) At day 20, most of the cells express mature hepatocyte markers such as albumin (ALB, *green*) and alpha-1 antitrypsin (AAT, *red*). Scale bars, 100 μm

immune staining. Based upon cell morphology and Sox17 expression, endoderm differentiation is not significantly influenced by alcohol exposure (Fig. 2a). Flow cytometry analysis demonstrates increased Annexin V-positive apoptotic cells after alcohol treatment (Fig. 2a). Cell proliferation shows little to no change at this stage (Fig. 3a).

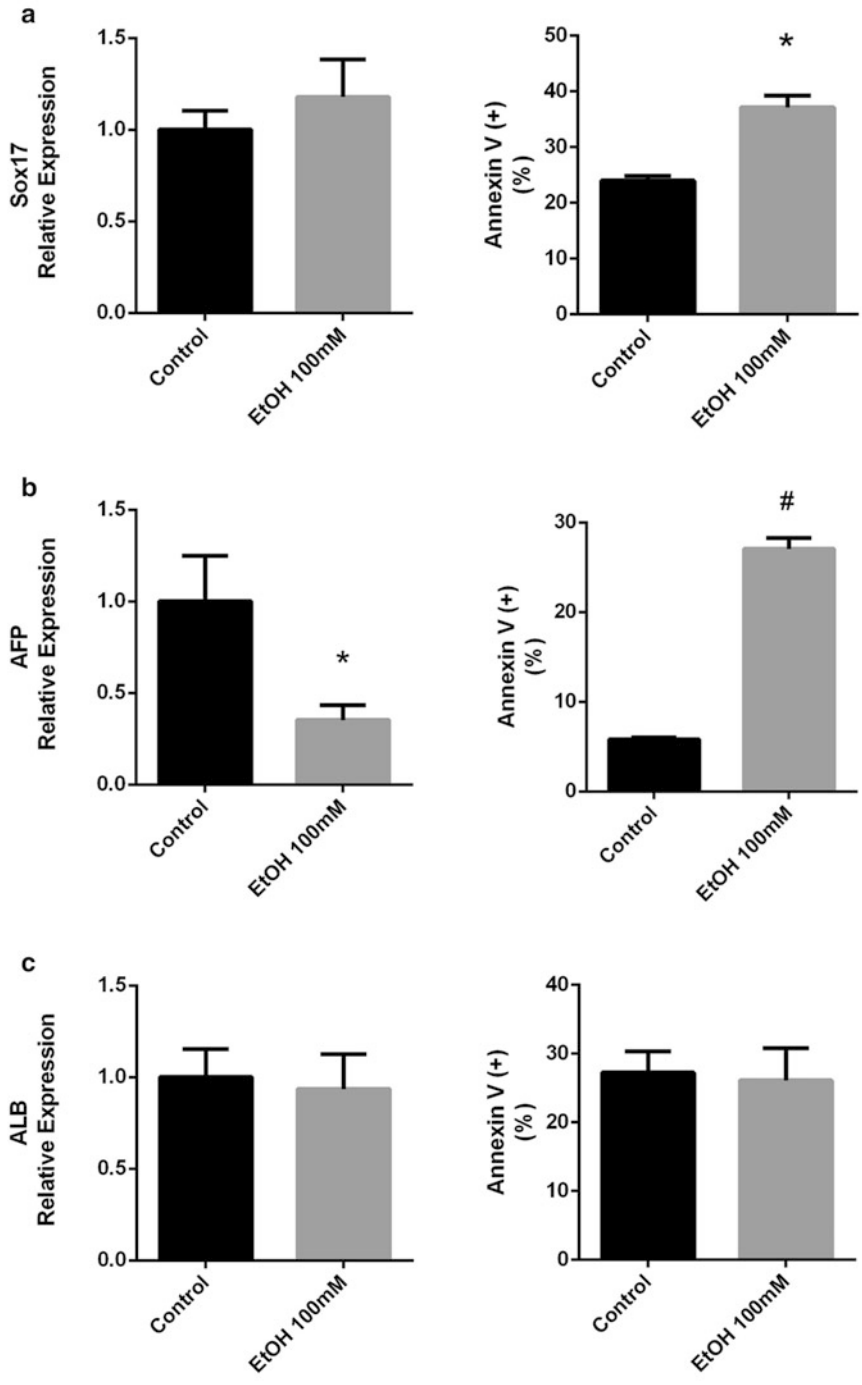

Fig. 2 Effects of alcohol on hepatic differentiation and cell viability. (**a**) Real-time PCR analysis of Sox17 expression (*left panel*) at day 4 definitive endoderm (DE) stage, following 100 mM ethanol treatment from day 0 to day 3. Percentage of Annexin V-positive cells was obtained from analysis of cell apoptosis by flow cytometry (*right panel*). Apoptotic cells were increased with alcohol exposure at DE stage. (**b**) Significant reduction in AFP expression (*left panel*) was observed in hepatic progenitor (HP) cells at day 9, on exposure to

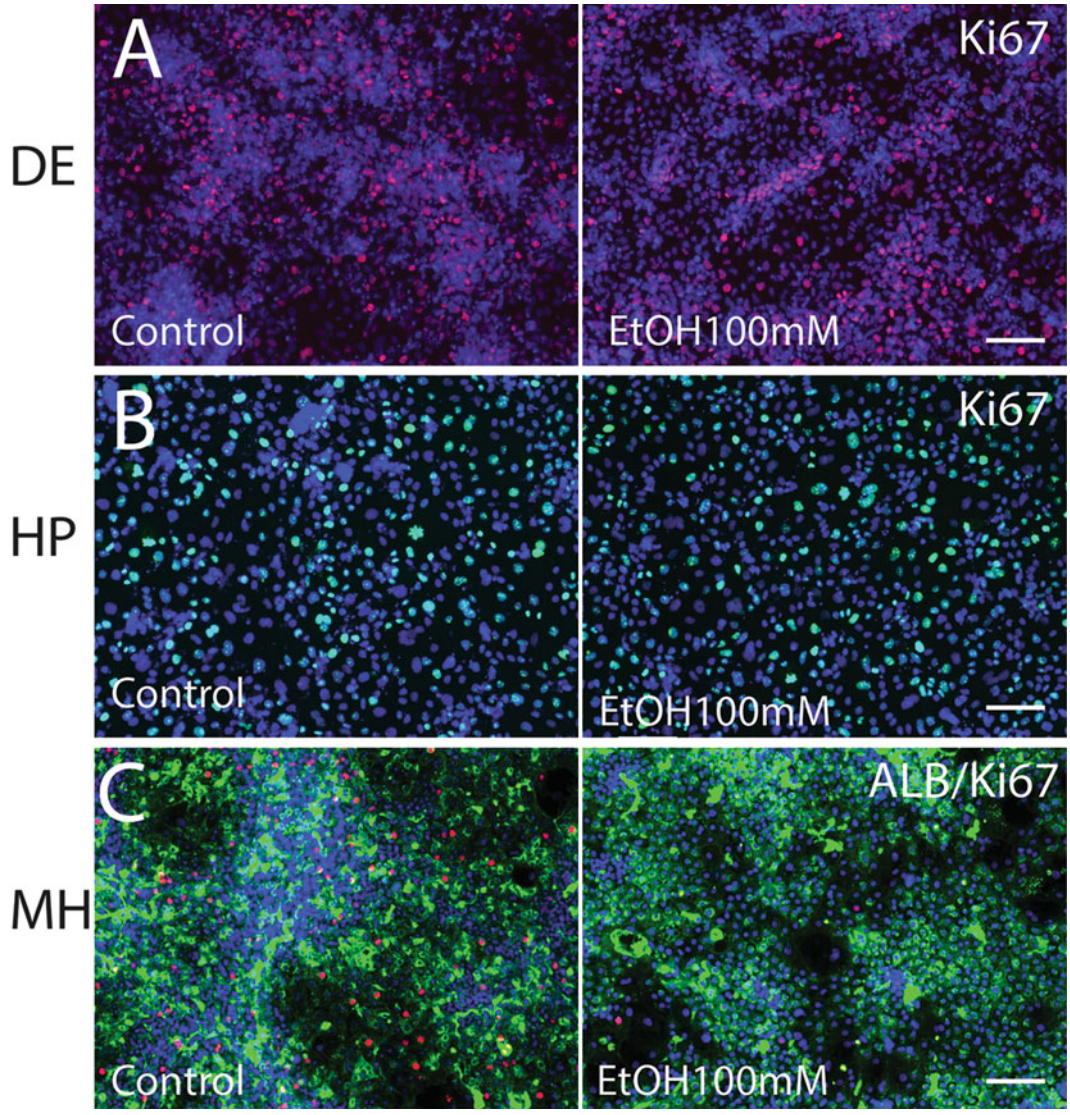

Fig. 3 Effects of alcohol on proliferation of iPSC-derived multistage hepatic cells. (**a**) Representative images of Ki67 (*Red*)-positive cells at day 4 DE stage, (**b**) Ki67 (*green*)-positive cells at day 9 HP stage, (**c**) ALB-positive cells (*green*), and Ki67 (*red*)-positive cells at day 16 MH stage in control and ethanol-treated cells. Scale bars, 100 μm

Fig. 2 (continued) 100 mM ethanol from day 4 to day 8, and the quantity of Annexin V-positive apoptotic cells also increased significantly (*right panel*). (**c**) Alcohol treatment from day 11 to day 15, followed by analysis of albumin (ALB) expression (*left panel*) and cell viability (*right panel*) of the mature hepatocyte-like cells at day 16, showed no statistical difference compared with control. *: $p < 0.05$, #: $p < 0.01$

2. Effects of alcohol on differentiation of iPSC-derived DE cells into hepatic progenitor (HP) stage cells: Differentiate iPSCs to HP stage cells, as described in Sect. 3.2. Treat the cells with 100 mM ethanol from day 4 to 8. At day 9, perform real-time PCR, apoptosis assay, and Ki67 staining (Figs. 2b and 3b). Hepatic progenitor marker, AFP, shows significant reduction after ethanol treatment. This is accompanied with significantly increased cell apoptosis at HP stage (Fig. 2b). Cell proliferation does not change significantly at this stage (Fig. 3b).
3. Effects of alcohol on differentiation of iPSC-derived HP cells into hepatocyte-like cells: In this section, treat the iPSC-derived early hepatic cells with ethanol from day 11 to 15. Measure the expression of hepatocyte-specific marker albumin at day 16, by real-time PCR (Fig. 2c). Maturation and viability of iPSC-derived hepatocytes is not significantly affected by ethanol treatment at this stage. However, Ki67-positive cells are remarkably reduced (Fig. 3c), which suggests that alcohol influences cell proliferation of iPSC-derived hepatocytes.

3.4 Estimation of Fatty Changes in iPSC-Derived Mature Hepatocytes on Alcohol Exposure

Liver steatosis is the most common form of ALD, which can be found in more than 80 % patients with chronic alcohol consumption.

1. Differentiate human iPSCs to mature hepatocyte-like cells (Section 3.2).
2. At day 20 of differentiation, a majority of the cells express mature hepatocyte markers ALB and AAT (Fig. 1e, f). Treat these cells with 100 mM ethanol in HCM supplemented with 2 % FBS, 10 ng/ml HGF, 10 ng/ml FGF-4, and 10 ng/ml OSM, from day 20 to 24. Ethanol containing culture medium is prepared freshly prior to treatment. Seal the culture plates with parafilm to limit ethanol evaporation and maintain ethanol concentration (21). Change medium daily and culture at 37 °C and 5 % CO_2.
3. At day 25, harvest the cells for real-time PCR or fix them using 4 % PFA for measurement of lipid accumulation (Fig. 4).
4. Staining of lipid droplet: Fix the cells with 4 % PFA at room temperature for 30 min. Wash the cells with PBS once and twice with ddH_2O. Incubate the cells in 60 % isopropanol for 5 min, followed by 60 % Oil Red O solution (diluted in ddH_2O) for 5 min. Finally, wash the cells with ddH_2O until excess stain is no longer apparent. Keep cells covered with ddH_2O at all times. Lipid droplets appear red under microscope (Fig. 4a, b).
5. Quantification of lipid accumulation: After Oil Red O staining, wash the cells thrice with 60 % isopropanol. Extract the Oil Red O by treating the cells with 100 % isopropanol for 5 min with

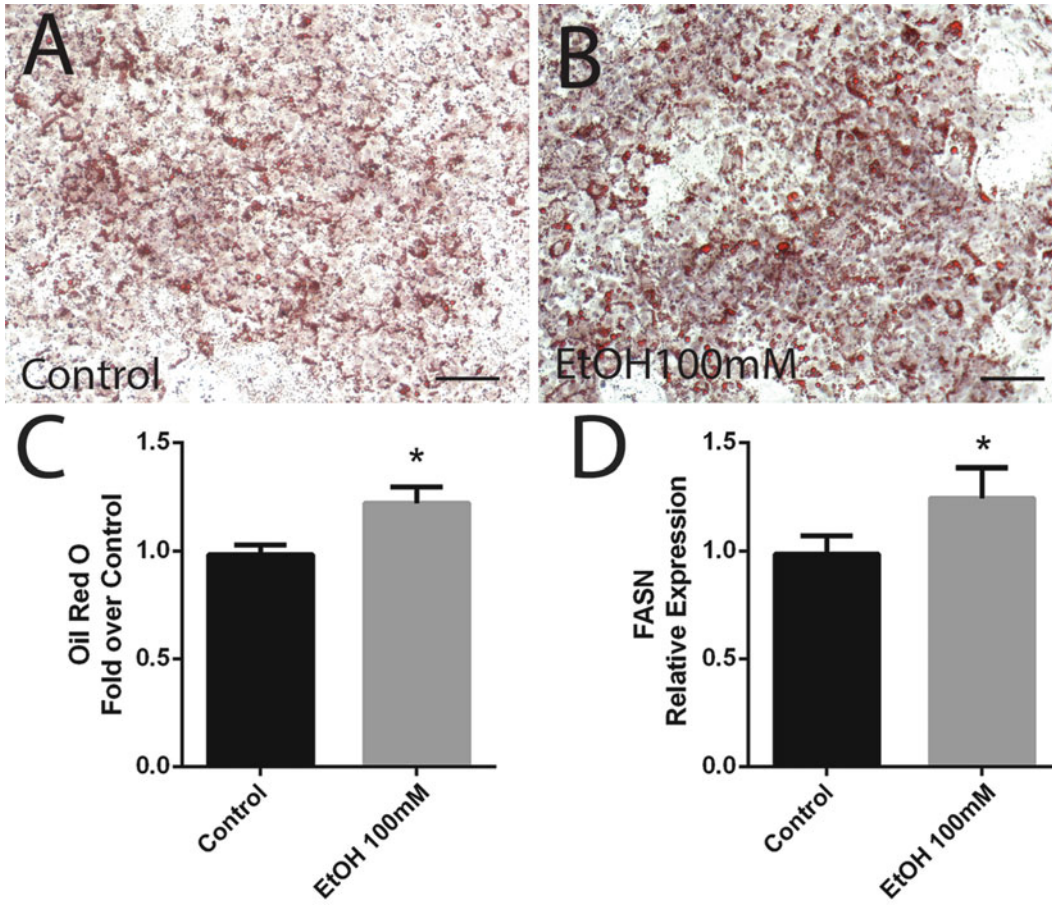

Fig. 4 Ethanol exposure induces fat accumulation in differentiated hepatocytes from iPSCs. (**a** and **b**) Detection of lipid droplets by Oil Red O. (**c**) Quantification of lipid accumulation with Oil Red O staining. (**d**) Real-time PCR analysis of expression levels of fatty acid synthase (FASN) in control and 100 mM ethanol-treated groups. *: $p < 0.05$. Scale bars, 100 μm

gentle rocking. Use 100 % isopropanol as background control when reading absorbance at 492 nm. Human iPSC-derived hepatocytes contain increased amount of lipid droplets after 100 mM alcohol treatment (Fig. 4c).

6. To further confirm alcohol-induced lipid accumulation, analyze the expression of fatty acid synthase (FASN) using real-time PCR. Cells treated with 100 mM alcohol show increased FASN transcription, when compared with the untreated control (Fig. 4d).

4 Notes

1. Use the cell culture plates within 1 week of coating with Matrigel. The plates should not be used for human iPSC/ESC culture, if not fully coated.
2. Freeze cells from 6 wells of a 12-well plate (approximately two to four million cells) in one freezing vial with CryoStem Freezing medium. When thawing, thaw the cells from one freezing vial into an entire 12-well plate. Do not start the hepatic differentiation right after thawing the iPSCs when the cells are still quiescent.
3. Pass the colonies as small cell aggregates. The split ratio varies with the growth condition of different cell lines (1:2–1:5). Y-27632 at 2–10 μM can be added to the culture medium to increase cell viability.
4. There is difference in terms of ingredients among RPMI 1640 medium from different suppliers. The RPMI 1640 medium from Gibco works better for endoderm differentiation of most of the iPSC lines tested by us.
5. Observe the cells under the microscope every day, and then determine an optimum concentration of B27 (0.02–2 %) based upon cell viability and differentiation status. Ideally, there should be cells with uniform DE morphology attached to the plate and less than 5 % of dead cells floating in the medium. When cell viability is compromised, increase B27 concentration to protect the cells. It is essential to keep an even distribution of the cells in the plates in order to obtain the best DE differentiation efficiency (>99 %).
6. Adding FBS can increase viability but is not necessary for hepatic differentiation or hepatic functionality. Therefore, FBS can be omitted for certain purposes including xeno-free human liver cell generation or experiments requiring serum-free conditions.

Acknowledgements

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Generation of Human Induced Pluripotent Stem Cells Using RNA-Based Sendai Virus System and Pluripotency Validation of the Resulting Cell Population

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Abstract

Human induced pluripotent stem cells (hiPSCs) provide a platform for studying human disease in vitro, increase our understanding of human embryonic development, and provide clinically relevant cell types for transplantation, drug testing, and toxicology studies. Since their discovery, numerous advances have been made in order to eliminate issues such as vector integration into the host genome, low reprogramming efficiency, incomplete reprogramming and acquisition of genomic instabilities. One of the ways to achieve integration-free reprogramming is by using RNA-based Sendai virus. Here we describe a method to generate hiPSCs with Sendai virus in both feeder-free and feeder-dependent culture systems. Additionally, we illustrate methods by which to validate pluripotency of the resulting stem cell population.

Keywords: hiPSC, Sendai, Pluripotency

1 Introduction

1.1 Human Pluripotent Stem Cells

The discovery of human pluripotent stem cells (hPSCs) has had a profound effect on various areas of science and has opened up extraordinary opportunities for establishment of model systems that would enhance our understanding of the disease mechanisms.

There are two main types of stem cells encompassed within the description of hPSC that are utilized in disease modelling, namely, embryonic and induced pluripotent cells. Mammalian embryonic stem cells (ESCs) are derived from pre-implantation blastocysts (1–3). Their defining features include the capability for indefinite undifferentiated proliferation in vitro and the potential to differentiate into cell types representative of the three embryonic germ layers (4). The potential of human ESCs (hESCs) has been explored extensively in studying development, disease modelling, drug screening, and therapeutics. On the other hand, controversy and ethical issues have arisen concerning their derivation and use. In addition, concerns have also been raised related to their potential to generate immunological and oncological barriers if used in therapy (5).

A major breakthrough was achieved with the discovery of hiPSCs, a potential alternative to hESCs. Two independent research groups reported reprogramming of terminally differentiated somatic cells through the ectopic expression of four transcription factors directing them to revert back to a pluripotent state (6, 7). HiPSCs were shown to be comparable to hESCs in many characteristics including their morphology, proliferation, pluripotency potential and epigenetic status. Since then, hiPSCs have become a popular tool to study human development in vitro, disease modelling, studying patient-specific mutations, and drug screening.

1.2 Reprogramming Human Somatic Cells

Yamanaka suggested two mechanistic models of reprogramming of somatic cells (8). He proposed the elite model of reprogramming which follows a deterministic approach suggesting that only a small proportion of cells is competent for reprogramming, a notion that has been supported experimentally by one study (9). The second model follows a view which supports the idea that reprogramming is a stochastic process and most somatic cells have the potential to become pluripotent. Indeed, Buganim et al. (10) showed that reprogramming follows early stochastic gene expression changes which is followed by a more deterministic phase. Another study combined the two hypotheses and used genome-wide analyses to suggest that a probabilistic phase is separated by early and late deterministic phases (11).

1.2.1 Reprogramming Methods

In 1938, Hans Spemann proposed that a differentiated nucleus can express pluripotency markers; but it was not until 1952 that Robert Briggs and Thomas King confirmed this theory experimentally when they successfully performed somatic cell nuclear transfer (12). They showed that transplanting nuclei from frog blastula cells into enucleated frogs' eggs results in normal embryonic development. John Gurdon extended these experiments by showing that nuclear transfer was also possible when transplanting nuclei from fully differentiated epithelial cells from *Xenopus* into enucleated eggs resulting in a small number of normal feeding tadpoles (13). These experiments were followed by a set of studies aimed at deriving cloned mammals. Initial mouse studies showed that transferring nuclei from eight-cell embryos to two-cell embryo cells resulted in normal embryonic development up to 12 days of gestation and in some cases to full term (14, 15). Later experiments indicated that it was possible to achieve viable offspring using cells derived from a sheep by transferring adult nuclei to enucleated oocytes (16, 17). Further studies showed the establishment of ESC lines by nuclear transfer from mouse and primate cells by using mature T-cells and adult skin fibroblasts donors (18–20). These studies illustrated the potential for future clinical applications; however the induction efficiency of this technique remained low. Recently, ESCs were

obtained using human cells by somatic cell nuclear transfer using female dermal fibroblasts of fetal origin and oocytes; however, the process, although relatively efficient, remains complex and is dependent on the availability of donated human oocytes (21).

In 2006, two independent research groups reported reprogramming human somatic cells into iPSCs by identifying a set of transcription factors in order to induce expression of pluripotency genes and chromatin modification (6, 7). Both of the groups used *SOX2* and *OCT3/4*. Additionally, Takahashi et al. (7) introduced *KLF4* and *c-MYC*, and Yu et al. (6) used *NANOG* and *LIN28*. The groups used retroviral vectors as a method of delivering the transcription factors. Since these first experiments, a number of different approaches have been established with the aim to achieve faster and more efficient reprogramming.

Viral vectors are commonly used to deliver target genes and they can either be integrating or non-integrating. Integrating viral vectors, including lentiviruses, have been shown to be efficient; however they carry a risk of promoting insertional mutagenesis and may induce tumorigenicity (22). In order to overcome some of these issues, alternative methods of reprogramming have been developed, including non-integrating viral and nonviral transgene delivery.

Nonviral Delivery

Transfection with non-integrating episomal vectors has been investigated as an alternative to the delivery of viral vectors. Several groups reported successful reprogramming of somatic cells using this method however the efficiency remains low (0.0003–0.0006 % for fibroblasts when reprogrammed with vectors containing *OCT4*, *SOX2*, *NANOG*, *LIN28*, *c-Myc*, *KLF4*, and *SV40LT*; 0.0007 % for newborn cord blood, and adult peripheral blood mononuclear cells when reprogrammed with *OCT4*, *SOX2*, *KLF4*, *c-MYC*, and *LIN28*; ~0.003 % for human dermal fibroblasts and dental pulp when reprogrammed with *OCT4*, *SOX2*, *KLF4*, *LIN28*, *MYCL1*, and *TP53*) (23–25).

Other groups have described generation of hiPSCs using nonviral minicircle DNA which is free of bacterial DNA. However, unlike transfection with episomal vectors, protocols using minicircle DNA require multiple transductions and still result in low reprogramming efficiency (~0.005 %) (26, 27).

Methods also exist that avoid using DNA in the reprogramming process. Recombinant cell-penetrating proteins Oct4, Sox2, Klf4, and c-Myc delivered to murine fibroblast cells have been shown to be able to induce pluripotency (28, 29). However, the process has been shown to be stochastic and in some protocols the use of subsequent transductions and addition of valproic acid was necessary leading to increased cell death and low efficiency (~0.001 %). Similar work with human somatic cells also showed the necessity of prolonged repeated protein treatment cycles with a

very low efficiency of hiPSCs generation (0.001 % efficiency when delivering OCT4, SOX2, KLF4, and c-MYC reprogramming proteins) (30).

One of the ways to overcome some of the difficulties associated with the use of proteins in reprogramming is to deliver reprogramming factors via messenger RNA. This allows post-translational modification of the proteins to occur inside the cells in natural environment. Although, this way of reprogramming eliminates some issues associated with other methods and potentially results in high reprogramming efficiency (2–4.4 %), it is labor-intensive and requires numerous daily transfections (31, 32).

Non-integrating Viral Vectors

Non-integrating viral vectors have been proposed in order to circumvent the risks of insertion of the DNA in a host genome. One such reprogramming technique is using an adenovirus which transiently expresses reprogramming factors predominantly without integrating into the host genome. This method of reprogramming allows transfection of non-dividing cells such as hepatocytes; however, the efficiency of such reprogramming technique is low (0.0002 %) and requires repeated transfections (33).

It is possible to achieve efficient transgene-free reprogramming using Sendai virus. This is a parainfluenza virus that belongs to the *Paramyxoviridae* family, first isolated in Japan in the 1950s from an infected mouse (34, 35). The Sendai viral particle is 150–200 nm in diameter. Its genome consists of 15,384 nucleotides and replicates as a negative-strand RNA. It was first developed in its inactive form retaining the ability to self-replicate by Li et al. (35). The system that we use contains three vectors, encoding *hKLF4*, *hc-MYC* and a polycistronic vector encoding *hKLF4*, *hOCT3/4*, *hSOX2*. The virus is capable of reprogramming various types of somatic cells including fibroblasts and T cells with efficiencies ranging between 0.1 and 0.2 % (36, 37). CD34+ cord blood cells have also been successfully reprogrammed (38). Sendai virus remains in the cytoplasm and does not have the ability to integrate into the host genome. Most commonly, virus clearance is achieved by clonal propagation of primary colonies leading to isolation of a sub-clone free of viral genome. Alternatively, it is possible to use temperature-sensitive vectors that enable virus clearing from the host cell after a number of passages (39).

Here we describe a method of deriving hiPSCs from human fibroblasts in feeder-free and feeder-dependent culture conditions and their subsequent characterization using the Sendai-based reprogramming system from Life Technologies (CytoTune®; www.lifetechnologies.com/order/catalog/product/A16517).

2 Materials

All the medium preparations should be performed in sterile conditions in a laminar flow tissue culture hood. Complete medium should be kept at 4 °C and pre-warmed to room temperature prior to use.

2.1 Medium and Solutions Composition

1. *Fibroblast medium*: Advanced DMEM (Dulbecco's Modified Eagle Medium), high glucose (Life Technologies), 10 % Fetal Bovine Serum (FBS) (Life Technologies), 1 % Penicillin–Streptomycin Solution (Life Technologies), 1 % GlutaMAX (Life Technologies).
2. *Matrigel coating*: BD Matrigel™ Basement Membrane Matrix (BD Biosciences). Use 0.5 mg of protein (as indicated in the data sheet with each product) diluted in DMEM/F-12 (Life Technologies) per 6-well plate. For coating steps, refer to the manufacturer's instructions.
3. *Complete mTeSR1 medium*: mTeSR™1 Basal Medium, mTeSR™1 5× Supplement (both STEMCELL Technologies), 1 % Penicillin–Streptomycin Solution (Life Technologies).
4. *Freezing medium*: 90 % FBS (Life Technologies), 10 % Dimethyl sulfoxide (DMSO) (Sigma), 10 μM Y-27632 (Chemdea). Prechilled at 4 °C.
5. *hiPSC medium* (for use in feeder-dependent culture): Knock-Out DMEM (Life Technologies), 20 % Knock-Out Serum Replacement (Life Technologies), 1 % MEM Nonessential Amino Acids Solution (Life Technologies), 1 % GlutaMAX (Life Technologies), 1 % Penicillin–Streptomycin Solution (Life Technologies), 8 ng/ml basic Fibroblast Growth Factor (bFGF) (Life Technologies).
6. *Conditioned mouse embryonic fibroblast* (*MEF*) *medium*: hiPSC medium conditioned by exposure to mitotically inactivated MEFs (*see* Section 3.2.2) supplemented with 1 % Insulin–Transferrin–Selenium (Life Technologies), 8 ng/ml bFGF, (Life Technologies) and filtered through a 0.2 μm filter. If not to be used straightaway, the medium can be stored at 4 °C for 2 weeks or alternatively at −20 °C for 3 months and supplemented right before use.
7. *Collagenase type IV solution*: Collagenase type IV (Life Technologies) powder dissolved in hiPSC medium to a concentration of 1 mg/ml. The solution should be filtered through a 0.2 μm filter. Solution aliquots can be stored at −20 °C for up to 6 months.
8. *Collagenase type IV and Dispase II solution*: 1:1 solution of Collagenase type IV (Life Technologies) powder dissolved in

hiPSC medium to a concentration of 1 mg/ml and Dispase II (Life Technologies) powder dissolved in Knock-Out DMEM (Life Technologies) to a concentration of 0.5 mg/ml. The solution should be filtered through a 0.2 μm filter.

9. *Embryoid body formation medium*: DMEM/F-12 (Life Technologies), 20 % FBS (Life Technologies), 1 % Penicillin–Streptomycin Solution (Life Technologies), 1 % MEM Nonessential Amino Acids Solution (Life Technologies).

2.2 Other Materials and Reagents

2.2.1 Reprogramming

1. CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Life Technologies) (*see* **Note 1**).

2.2.2 Cell Culture

1. Dulbecco's phosphate-buffered saline (DPBS), no calcium, no magnesium (Life Technologies).
2. 0.05 % Trypsin–EDTA (1×) solution (Life Technologies).
3. Versene® (EDTA) 0.02 % solution (Lonza).
4. Porcine gelatin (Sigma), 0.1 % prepared in DPBS and filtered when warm.
5. StemPro® Accutase® Cell Dissociation Reagent (Life Technologies).

2.2.3 Sendai Virus Clearance and Quantitative Polymerase Chain Reaction (PCR)

1. ReliaPrep™ RNA Cell Miniprep System (Promega).
2. GoScript™ Reverse Transcription System (Promega).
3. GoTaq® DNA Polymerase kit (Promega).
4. Agarose (Lonza).
5. GelRed™ (Biotium).
6. 100 bp step ladder (Promega).
7. UV Transilluminator (Gel Doc-II Imaging system).

Table 1
List of primers for the Sendai clearance experiment

Target	Primer sequence	Product size
SeV	Forward: GGA TCA CTA GGT GAT ATC GAG C Reverse: ACC AGA CAA GAG TTT AAG AGA TAT GTA TC	181 bp
KOS	Forward: ATG CAC CGC TAC GAC GTG AGC GC Reverse: ACC TTG ACA ATC CTG ATG TGG	528 bp
KLF4	Forward: TTC CTG CAT GCC AGA GGA GCC C Reverse: AAT GTA TCG AAG GTG CTC AA	410 bp
c-MYC	Forward: TAA CTG ACT AGC AGG CTT GTC G Reverse: TCC ACA TAC AGT CCT GGA TGA TGA TG	532 bp

Table 2
List of primers for identification of endogenous pluripotency genes experiment

Target	Primer sequence	Product size
GAPDH	Forward: GGCATGGACTGTGGTCATGAG Reverse: TGCACCACCAACTGCTTAGC	87 bp
KLF4	Forward: ACCCACACAGGTGAGAAACCTT Reverse: GTTGGGAACTTGACCATGATTG	313 bp
SOX2	Forward: TCACATGTCCCAGCACTACC Reverse: CCCATTTCCCTCGTTTTTCT	137 bp
OCT4	Forward: GAGGAGTCCCAGGACATCAA Reverse: CATCGGCCTGTGTATATCCC	100 bp
NANOG	Forward: CCAAATTCTCCTGCCAGTGA Reverse: CAGGTGGTTTCCAAACAAGAA	260 bp

8. For Sendai virus clearance primers *see* Table 1.
9. GoTaq® qPCR Master Mix (Promega).
10. hiPSC characterization primers are listed in Table 2.
11. 7900HT Fast Real-Time PCR system (Applied Biosystems).
12. qBase software.

2.2.4 Flow Cytometry

1. 2 % paraformaldehyde (PFA) dissolved in DPBS.
2. Methanol (prechilled at −20 °C).
3. *Incubation buffer*: Dissolve 0.5 g of bovine serum albumin (BSA) (Sigma) in 100 ml 1×DPBS. Store at 4 °C.
4. *Antibodies*: Rabbit mAb (Alexa Fluor® 647 Conjugate), Nanog (D73G4) XP® Rabbit mAb (Alexa Fluor® 647 Conjugate) (both Cell Signaling Technology), Alexa Fluor® 488 Mouse IgM, Alexa Fluor® 488 anti-human TRA-1-60-R (both BioLegend).

2.2.5 Immunocytochemistry

1. 4 % PFA dissolved in DPBS.
2. 0.25 % Triton X-100 (Sigma) prepared in DPBS.
3. *Blocking solution*: 10 % FBS (Life Technologies) and 1 % BSA (Sigma) in DPBS
4. *Antibodies*: Alexa Fluor® 555 Mouse anti-SSEA-4 (BD Biosciences), Goat anti-human Oct-3/4 Affinity Purified Polyclonal Ab (R&D Systems), Anti-Goat IgG (whole molecule)–FITC antibody (Sigma).
5. CyStain® DNA (DAPI) (PARTEC).
6. Fluorescence microscope.

2.2.6 Embryoid Body Formation

1. 3-Germ Layer Immunocytochemistry Kit (Life Technologies).

2.2.7 Single Nucleotide Polymorphism (SNP) Array

1. QIAamp DNA Mini Kit (Qiagen).

2.2.8 Other

1. Faxitron CP-160 radiation machine.
2. NanoDrop instrument.
3. Mr. Frosty™ Freezing Container (Thermo Scientific).

3 Methods

All the work should be carried out in a laminar flow tissue culture hood equipped with a stereomicroscope using sterile techniques. Cells should be maintained in a humidified tissue culture incubator (37 °C, 5 % CO_2, 20 % O_2).

3.1 Feeder-Free Reprogramming of Human Fibroblasts

The experimental timeline for reprogramming in feeder-free conditions is illustrated in Fig. 1.

1. On Day 1, prepare the fibroblasts for transduction by plating them on two wells (one well to be used as a negative control) of a 12-well plate at a density of 1×10^5 cells per well.
2. Culture the cells for 24 h in Fibroblast medium ensuring that the cells attached fully.
3. On Day 2 perform the transduction. Warm 1 ml of the Fibroblast medium per one well to be transduced. Thaw the aliquots of virus one at a time and add the appropriate volume to the medium. Pipette up and down to ensure the solution is thoroughly mixed. Perform the next step in 5 min.

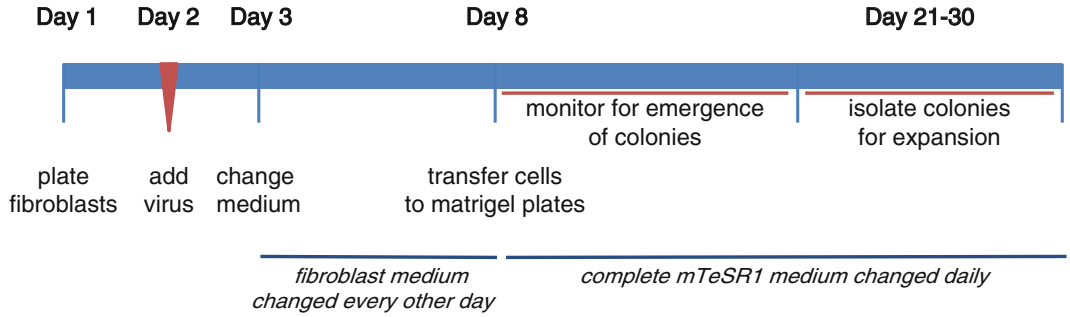

Fig. 1 Experimental timeline for feeder-free reprogramming of human fibroblasts

4. Aspirate the medium from the cells and add 1 ml of the Fibroblast medium containing the virus to each well to be transfected.
5. Place the cells in an incubator overnight.
6. On Day 3, replace the medium with 1 ml of fresh Fibroblast medium (*see* **Note 2**).
7. Replace the medium every other day until Day 7.
8. Prepare Matrigel plates to be used on Day 8.
9. On Day 8, cells are ready to be passaged. Aspirate the medium and wash the cells with warm DPBS. Add 500 µl of pre-warmed 0.05 % Trypsin–EDTA (1×) solution per well and incubate for ~3 min at 37 °C monitoring closely to note when the cells start rounding up and lifting. Try not to increase the incubation time unnecessarily. Once most of the cells are detached, add 2 ml of the Fibroblast medium and transfer the cells to a 15 ml conical tube. Centrifuge the cell suspension for 3 min at 300 × *g* at room temperature. Aspirate the medium and resuspend the cells in ~3 ml complete mTeSR1 medium. Count the cells using preferred method and re-plate the cells in complete mTeSR1 medium at a density of 5×10^4 cells per well of a 6-well plate. Perform full medium changes daily with 2 ml of medium per well. Complete mTeSR1 medium is to be used in all subsequent feeder-free cell culture. Freeze the remaining cells in the Freezing medium to be used as a positive control for the Sendai clearance test (Section 3.4). To cryopreserve the cells, centrifuge the remaining cells for 3 min at 300 × *g* at room temperature, resuspend in 1 ml of the Freezing medium, transfer to a labelled cryovial and freeze by placing it into a Mr. Frosty freezing container at −80 °C.
10. From Day 9 to Day 30 cells should be monitored under a microscope every other day for changes in morphology and first appearance of cell clumps indicating reprogramming.
11. Depending on the cell line, starting from Day 21 post-transduction, hiPSC colonies should grow to the desired size for transfer (*see* **Note 3**). Initial identification of the reprogrammed cells can be carried out on morphological basis. Colonies are tightly packed with cells having large nuclei to cytoplasm ratio (*see* Fig. 2).
12. For colony transfer, prepare 24-well plates coated with Matrigel to be used on the day (*see* **Note 4**). Allow one well of a plate per colony.
13. Aspirate Matrigel from the plate and add 500 µl of the complete mTeSR1 medium.
14. Identify colonies to be transferred by observing them under the microscope.

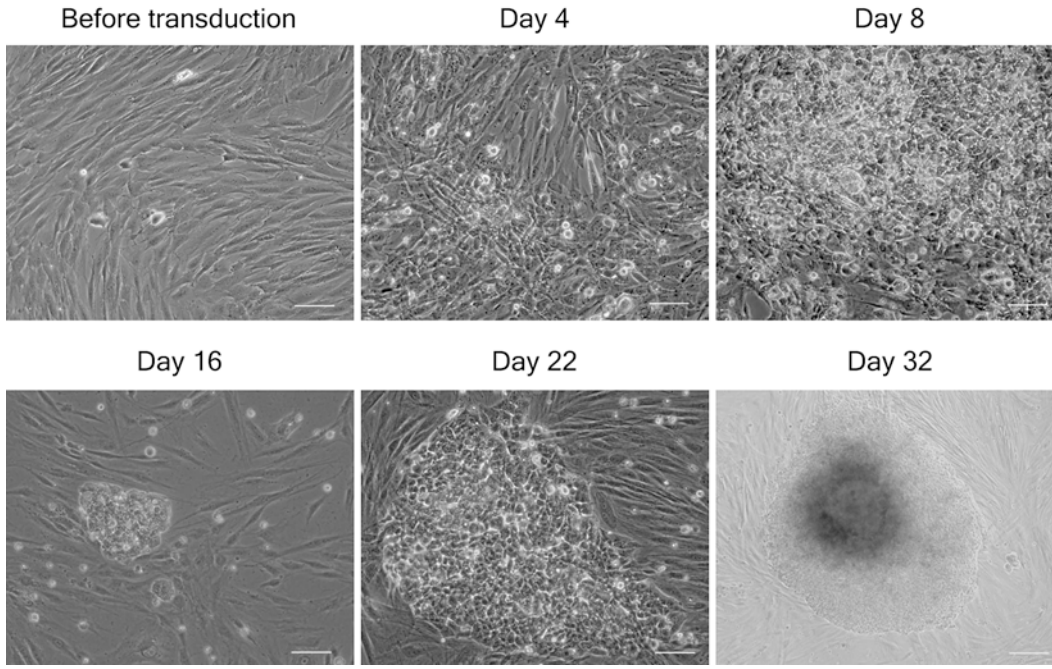

Fig. 2 Representative images of cells at different stages of reprogramming in feeder-free culture. Scale bar 100 μm

15. Cut them in five to six pieces using a 20 gauge needle or a P10 filter tip. Transfer the colony fragments using a 10 μl pipette.
16. Allow the colonies to attach for 24 h and change medium every day thereafter during expansion.
17. When expanding the resulting hiPSC population, perform the first passage at a ratio of 1:1.
18. Prepare 24-well Matrigel plates to be used on the day.
19. Aspirate Matrigel from the plate and add 500 μl of the complete mTeSR1 medium.
20. Identify colonies to be transferred and cut them in five to six pieces using a 20 gauge needle or a P10 filter tip. Transfer the colony fragments using a 10 μl pipette into the appropriate plates.
21. Allow the colonies to attach for 24 h and change the medium every day thereafter.
22. Monitor cells daily. Clean off differentiating cells with a P10 tip or a 20 gauge needle under the microscope.
23. When colonies are large enough, manually transfer them to fresh 12-well plates by cutting them in small pieces and transferring them with a P10 pipette at a ratio of 1:1.

24. Monitor the colonies daily for possible differentiation and remove differentiated cells.
25. When colonies are dense and large enough (every 5–7 days for good clones) passage the cells at a ratio of 1:2 on a 12-well plate twice. Subsequently passage at a ratio of 2:1 on a 6-well plate by which point the cells will be at P5. From the next passage onwards, clones can be passaged at a ratio of 1:3.
26. When the cultures are established, EDTA mediated passaging can be introduced (around P8-P11).
27. Colonies can be passaged with Versene® (EDTA) 0.02 % solution. Wash cells with Versene® (EDTA) 0.02 % solution or DPBS, add 1 ml of Versene® (EDTA) 0.02 % solution per well and incubate at room temperature for ~2–5 min. Colonies should start dissociating but not lifting completely. Aspirate Versene® (EDTA) 0.02 % solution and carefully add complete mTeSR1 medium holding the pipette tip perpendicular to the plate trying to avoid breaking up the colonies. Collect floating cells and transfer to a pre-warmed Matrigel plate. The ratio of passaging and therefore the amount of medium added during passaging will depend on the rate of growth of the cells and could range from 1:3 to 1:10 for more established cultures (*see* **Note 5**).

3.2 Feeder-Dependent Reprogramming of Human Fibroblasts

Some cell lines are more difficult to reprogram in feeder-free conditions. In this case it is recommended to do the reprogramming using a MEF feeder layer instead of feeder-free following the same timeline as used in the feeder-free protocol.

3.2.1 Preparation of MEF Feeder Layer

1. MEFs are isolated from 12.5 to 13.5 day old mouse embryos as described previously (40). For maintenance and expansion we use the Fibroblast medium recipe described in this protocol.
2. MEFs are cultured in T75 flasks at a density of 13–15 $\times$ 10^4 cells per flask.
3. Once confluent, the MEFs are inactivated by X-irradiation using a Faxitron CP-160 radiation machine at a dose of 120 kV, 4.0 mA for 7 min.
4. Let the cells recover in an incubator for 1 h post-inactivation then harvest using 0.05 % Trypsin–EDTA (1×) solution as described in Section 3.1.
5. The irradiated MEFs are plated on tissue culture plates pre-coated with gelatin at a density of 1.5 $\times$ 10^4 cells/cm^2. To prepare the plates, incubate them for 1 h at 37 °C or for 2 h at room temperature. Aspirate the gelatin and plate the cells in Fibroblast medium.
6. Allow the cells to attach for 24 h prior to use.

3.2.2 Preparation of MEF Conditioned Medium

1. When 80–90 % confluent, proliferating MEFs are irradiated as described in Section 3.2.1 in order to prevent further proliferation.
2. Let the cells recover in an incubator for 1 h post-inactivation.
3. The cells are then lifted with 0.05 % Trypsin–EDTA (1×) solution as described in Section 3.1, resuspended in Fibroblast medium and counted using the desired method.
4. Cells are then re-plated at a density of 5.6×10^4 cells/cm^2 in Fibroblast medium.
5. On the following day, the medium is changed to hiPSC medium by adding the appropriate volume (10 ml per T25 flask, 30 ml per T75 flask, and 120 ml per T300 flask).
6. The medium is then collected every day for 9 days, each day replacing it with fresh hiPSC medium.
7. Prior to use, the medium is supplemented as described in Section 2.1.

3.2.3 Feeder-Dependent Reprogramming

Experimental timeline for the reprogramming in feeder-dependent conditions is illustrated in Fig. 3.

1. On Day 1, prepare the fibroblasts for transduction by plating them on two wells (one well to be used as a negative control) of a 12-well plate at a density of 1×10^5 cells per well.
2. Culture the cells for 24 h in Fibroblast medium ensuring that the cells attached fully.
3. On Day 2 perform the transduction. Warm 1 ml of the Fibroblast medium per one well to be transduced. Thaw the aliquots with the virus one at a time and add the appropriate volume to the medium. Pipette up and down to ensure the solution is thoroughly mixed. Perform the next step in 5 min.
4. Aspirate the medium from the cells and add 1 ml of the Fibroblast medium containing the virus to each well to be transfected.

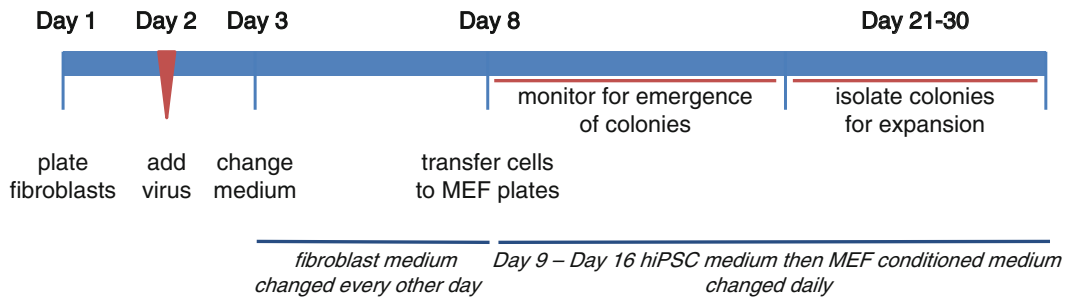

Fig. 3 Experimental timeline for feeder-dependent reprogramming of human fibroblasts

5. Place the cells in an incubator overnight.
6. On Day 3, replace the medium and change it every other day thereafter until Day 7 (*see* **Note 2**).
7. On Day 7, prepare the MEF plates. We recommend using 6-well plates.
8. On Day 8, aspirate the medium from MEF plates and add 1 ml of hiPSC medium per well and keep in an incubator for 30 min before use. Passage transduced fibroblasts with 0.05 % Trypsin–EDTA (1×) solution as described in Section 3.1. Count the cells and re-plate on MEF plates in 1 ml hiPSC medium at a density of 8×10^3 cells per well of a 6-well plate (*see* **Note 6**). Freeze the remaining cells in the Freezing medium to be used as a positive control for the Sendai clearance test (*see* Section 3.1 for the details on the freezing method and Section 3.4 for the Sendai clearance test). Perform full medium changes daily with hiPSC medium from Day 9 to Day 16. From Day 16 until colony transfer use MEF conditioned medium.
9. From Day 9 to Day 30 cells should be monitored under a microscope every other day for changes in morphology (*see* Fig. 4).
10. Depending on the cell line, starting from Day 21 post-transduction, cell colonies should grow to the desired size for

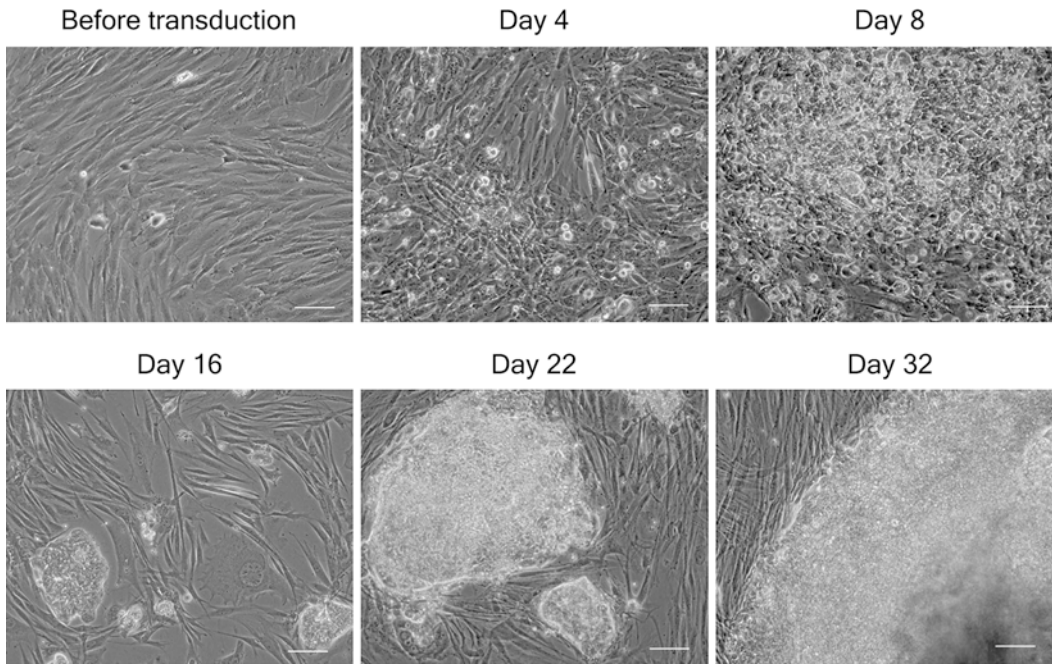

Fig. 4 Representative images of cells at different stages of reprogramming in feeder-dependent culture. Scale bar 100 μm

transfer (*see* **Note 3**). Prepare 24-well MEF plates 1 day before the transfer.

11. When expanding the resulting hiPSC population, perform the first passage at a ratio of 1:1.
12. Prepare 24-well MEF plates to be used on the day (*see* **Note 4**).
13. Change the medium to conditioned MEF medium for the first passage. For all the following passages, hiPSC medium can be used for the first 7 days. If keeping the colonies on the same plate for a longer period of time, switch the medium to MEF conditioned medium until the next passage.
14. Identify colonies to be transferred by observing them under the microscope and cut them in five to six pieces using a 20 gauge needle or a P10 filter tip. Transfer the colony fragments using a 10 μl pipette into the appropriate plates.
15. Allow the colonies to attach for 24 h and change the medium every day thereafter.
16. Monitor cells daily. Clean off differentiating cells with a P10 tip or a 20 gauge needle under the microscope.
17. When colonies are large enough, manually transfer them to fresh 12-well plates by cutting them in small pieces and transferring them with a P10 pipette at a ratio of 1:1.
18. Daily keep monitoring the colonies for possible differentiation and remove differentiated cells.
19. When colonies are dense and large enough (every 5–7 days for good clones) passage the cells at a ratio of 1:2 on a 12-well plate twice. Subsequently passage at a ratio of 2:1 on a 6-well plate by which point the cells will be at P5. From the next passage onwards, clones can be passaged at a ratio of 1:3.
20. When the cultures are established, enzymatic passaging can be introduced (around P8-P11).
21. Colonies can be passaged at a ratio of 1:3 with Collagenase type IV solution (*see* **Note 5**). Wash the cells with DPBS, add Collagenase IV solution for 2–8 min (until colony edges start lifting), aspirate the solution, add hiPSC medium and use P100 to separate the colonies. Transfer to a fresh MEF plate.

3.3 Adapting hiPSCs to Feeder-Free Conditions

We recommend using hiPSCs cultured in feeder-free conditions for all the downstream analyses. Feeder-dependent cultures could be adapted to feeder-free environment by following the protocol below.

1. 24 h prior to passaging, change the hiPSC medium to MEF conditioned medium.
2. Prepare Matrigel plates to be used on the day of transfer.

3. On the day of transfer, select the best looking well with well-defined hiPSC colonies. Remove differentiated cells.
4. Wash the cells with DPBS, add Collagenase IV solution and incubate for 2–8 min. Remove the solution and add MEF conditioned medium. Gently lift the colonies using a cell scraper trying not to break up the colony into small parts.
5. Keep one well as a backup. If it is not possible to leave an entire well, leave some material on the well to serve as a backup.
6. Collect the cells into a falcon tube and rinse with MEF conditioned medium.
7. Centrifuge at 300 × *g* for 3 min.
8. Resuspend in the required volume of MEF conditioned medium and plate the cells on Matrigel coated plates. Place in an incubator overnight allowing the colonies to attach.
9. The next day change the medium to complete mTeSR1 medium and feed every day (*see* **Note 7**).

3.4 Sendai Virus Clearance

We recommend testing for Sendai virus clearance from the established clones starting from P7 onwards. The procedure involves RNA extraction, cDNA synthesis using Reverse Transcriptase and DNA polymerization. All the RNA work should be performed in an RNase-free environment.

1. Harvest the cells grown in feeder-free conditions with Versene® (EDTA) 0.02 % solution into a conical 15 ml tube. Use the cells frozen on Day 8 of the reprogramming as a positive control.
2. Centrifuge the cells at 300 × *g* for 3 min.
3. Wash the cells with DPBS and centrifuge again to obtain a cell pellet.
4. Extract RNA using ReliaPrep™ RNA Cell Miniprep System according to the manufacturer's instructions.
5. The purity of RNA can be tested with a NanoDrop instrument expecting the ratio of A_{260}/A_{280} to be in the region of 1.8–2.2.
6. cDNA preparation is performed using GoScript™ Reverse Transcription System according to the manufacturer's instructions.
7. Perform DNA polymerization using GoTaq® DNA Polymerase kit following the instructions of the manufacturer. PCR program: denaturation 94 °C 30 s, annealing 55 °C 30 s, elongation 72 °C 30 s for 30 cycles.
8. Analyze the PCR product using 2 % agarose gel electrophoresis using a 100 bp step ladder. See the list of primers in Table 1.
9. Run the gel at 90 V for 1 h and 30 min.
10. The gel can be visualized for the appearance of bands with a UV Transilluminator (230 V–50 Hz) (*see* Fig. 5).

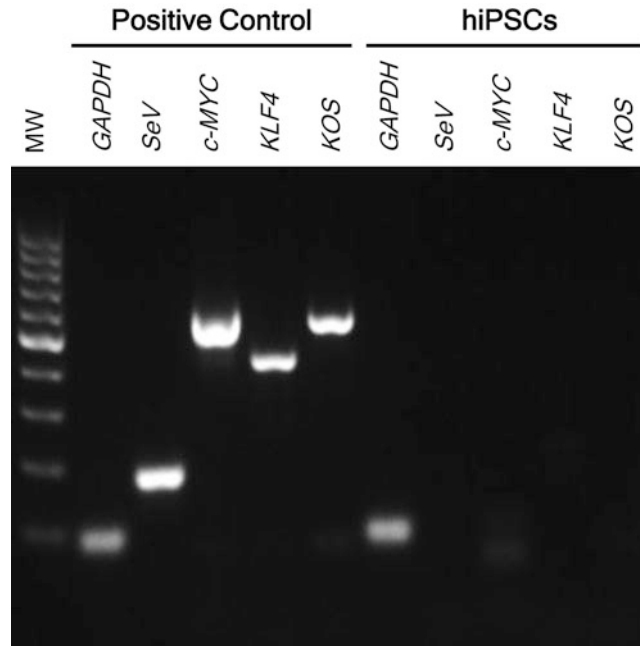

Fig. 5 Representative images of the gel showing the PCR product confirming absence of Sendai vectors in the established population of hiPSCs

3.5 Characterization of the Resulting hiPSC Population

A range of techniques is applied in order to validate pluripotency. Firstly, observe the morphology of the cells and confirm their resemblance to embryonic stem cells characterized by their cobblestone appearance, tight flat colonies, and large nuclei. This is followed by a number of molecular and functional tests outlined below.

3.5.1 Quantitative PCR for Identification of Endogenous Pluripotency Genes KLF4, SOX2, OCT4, and NANOG

1. Isolate RNA and synthesize cDNA as described in Section 3.4.
2. Perform quantitative PCR using GoTaq® qPCR Master Mix following the instructions of the manufacturer. We recommend the following settings: hot start activation 95 °C for 2 min 1 cycle, denaturation 95 °C for 15 s, annealing/extension 60 °C for 60 s 40 cycles, dissociation 60–95 °C. Primers are listed in Table 2.
3. Detection and quantification of the sequences of interest is performed by the 7900HT Fast Real-Time PCR system. The relative expression values for hESCs are used as reference values.
4. Relative quantification of the genes can be studied using qBase software.

3.5.2 TRA-1-60 and NANOG Characterization by Flow Cytometry

1. Prepare single-cell suspension using Accutase solution. Wash the cells with DPBS, aspirate and add 0.5 ml of Accutase per well of a 6-well plate. Incubate at 37 °C for 2–3 min until the cells detach. Stop the reaction by diluting Accutase in 5 mM EDTA in DPBS. Collect the cells into a 15 ml conical tube and centrifuge at 300 × *g* for 5 min at 4 °C.
2. Aspirate and add 10 ml of DPBS.
3. Centrifuge at 300 × *g* for 5 min at 4 °C.
4. Aspirate the supernatant and fix the cells with 2 % PFA for 10 min at 37 °C.
5. Add 5–10 ml of DPBS.
6. Centrifuge at 300 × *g* for 5 min at 4 °C.
7. Resuspend in 1 ml of methanol and incubate at −20 °C for 30 min (at this stage the cells can be either kept in the freezer for prolonged storage or you can proceed to staining on the same day).
8. Add 5–10 ml of DPBS and count the cells.
9. Centrifuge at 300 × *g* for 5 min at 4 °C.
10. Remove the supernatant and add the incubation buffer to obtain 2–5 × 10^5/200 μl cell suspension.
11. Add 200 μl of cell suspension to each well of a 96-well plate with U-shaped bottom.
12. Centrifuge the plate at 200 × *g* for 5 min at 4 °C. Remove the supernatant by flicking the whole plate with the cells still attached to the bottom of the plate.
13. Add isotype control Alexa Fluor 647 to one well and Alexa Fluor 647 conjugated anti-Nanog antibody to another well at the concentration of 1:150. Gently resuspend. Alternatively, add isotype control Alexa Fluor 488 to one well and Alexa Fluor 488 conjugated anti-TRA-1-60 antibody to another well at a concentration of 1:300.
14. Incubate for 1 h at room temperature.
15. Rinse with the incubation buffer as described previously.
16. Resuspend the cells in 0.5 ml of incubation buffer and perform flow cytometry analyses (*see* Fig. 6).

3.5.3 Immunocytochemistry for the Detection of SSEA-4 and OCT4

1. Choose two wells of a plate to be used for immunocytochemistry experiments.
2. Aspirate the medium and add 2 ml of DPBS per well of a 6-well plate.
3. Aspirate and fix the cells with 1 ml of 4 % PFA for 15 min at room temperature.

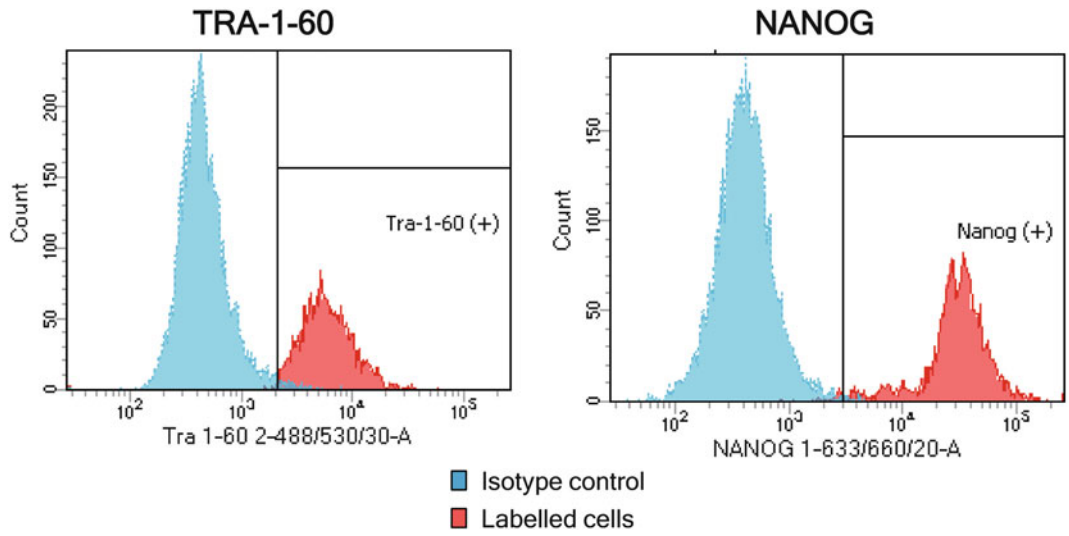

Fig. 6 Representative graphs illustrating flow cytometry results confirming the expression of pluripotency markers TRA-1-60 and NANOG in the population of hiPSCs

4. Aspirate and wash the well 3 times with DPBS.
5. Aspirate and permeabilize the cells with 1 ml of 0.25 % Triton X-100 for 40 min at room temperature.
6. Wash the cells with DPBS.
7. Add 2 ml of blocking solution and incubate for 45 min at room temperature.
8. Aspirate.
9. In one well add Alexa Fluor 555 conjugated anti-SSEA-4 antibody diluted 1:100 in blocking solution. In another well add goat anti-human Oct-3/4 primary antibody at a concentration of 1:100 in blocking solution, followed by incubation with a secondary antibody anti-goat IgG—FITC antibody at a concentration of 1:200. All the antibodies are diluted in blocking solution. Incubation time should be 1 h at room temperature for all the antibodies.
10. Aspirate the antibodies and wash the wells with DPBS three times, 3 min at a time.
11. Counterstain the nuclei with DAPI.
12. Aspirate and wash the wells with DPBS three times, 3 min at a time.
13. Submerge the cells in DPBS and image with the appropriate fluorescence microscope.

3.5.4 Embryoid Body Formation

This is a functional test to validate the ability of the resulting hiPSC population to spontaneously differentiate into cell types representative of the three embryonic germ layers.

1. For this experiment, use two 6-well plates with 20–30 hiPSC colonies per well.
2. Aspirate the medium and add 2 ml of DPBS per well of each plate.
3. Aspirate and add 1 ml of Collagenase type IV and Dispase II solution per well and incubate for 40 min at 37 °C checking for colony detachment.
4. Once the colonies have detached, collect the fragments/cells into a 50 ml conical tube and let them settle.
5. Aspirate the supernatant and resuspend the cells in 24 ml of Embryoid body formation medium.
6. Transfer the cells to a 10 cm Petri dish and keep in an incubator for 7 days changing the medium with Embryoid body formation medium every day.
7. On Day 8, transfer the embryoid bodies to a 24-well plate precoated with gelatin as described in Section 3.2.1. Replace the medium daily.
8. On Day 8, fix and stain the colonies for the ectodermal marker beta-III tubulin, mesodermal smooth muscle actin, and endodermal alpha-fetoprotein according to the instructions provided with the 3-Germ Layer Immunocytochemistry Kit.

3.5.5 SNP Array

The process of reprogramming has a potential of causing *de novo* mutations and chromosomal abnormalities in the resulting population of hiPSCs. It is therefore recommended that the cells are tested for any potential genetic abnormalities. Additionally, their genetic identity and origin should be confirmed by comparing them to the original fibroblasts they were derived from. Many commercial organizations, such as Oxford Gene Technology (www.ogt.co.uk) perform these analyses upon receipt of DNA. One of the methods that we recommend for performing the assay is Infinium HD Assay Ultra (Illumina). The basic principle of the method is outlined below.

1. DNA isolation is performed in-house with QIAamp DNA Mini Kit following the instructions of the manufacturer.
2. DNA content can be verified with a NanoDrop instrument.
3. 200 ng of DNA is to be sent to the organization performing the array. Minimum concentration 50 ng/μl. The exact instructions on the volume and concentration of the sample to be provided by the organization performing the assay.
4. Infinium HD Assay Ultra consists of the following steps;

(a) DNA amplification and fragmentation

(b) DNA fragment hybridization to a probe on the array

- The probe binds to a complementary sequence on the DNA and stops at the site of the variant

(c) The probe is extended with labelled nucleotides matching the target DNA sequence

(d) The nucleotide label is excited by a laser which causes the signal to emit detected by the scanner which results in the identification of the allelic ratio for a specific locus

(e) The data is then analyzed using GenomeStudio® software (Illumina)

4 Notes

1. CytoTune®-iPS 2.0 Sendai Reprogramming Kit consists of three vials containing polycistronic hKLF4–hOCT3/4–hSOX2 (KOS), hc-MYC, and hKLF4 vectors. The kit should be stored at −80 °C. If it is to be used for multiple transductions performed on different days aliquots can be made to avoid repeated freeze thawing. The aliquot size should be determined by calculating the amount of each vector needed to transfect 1×10^5 cells at a multiplicity of infection (MOI) as follows: KOS MOI = 5, hc-MYC MOI = 5, hKLF4 MOI = 3 (use titre information provided on the data sheet). All the work should be performed strictly on ice and the aliquots should be transferred to −80 °C immediately.
2. It is normal to observe some cytotoxicity the day after the transduction.
3. For difficult lines MOI could be adjusted by first increasing the MOI of hKLF4. Should that fail, try also increasing the MOI of hc-MYC.
4. For some cell lines, longer time periods are required to produce first colonies, therefore it is recommended to keep them in culture for up to 6 weeks. Try to transfer them to new plates before they start differentiating. Should that occur, dissect off differentiating cells prior to transferring. Do not pick colonies before Day 14 as they might not be fully reprogrammed.
5. If colonies do not grow at a desired rate, they can be passaged back to a smaller surface area until they grow confluent. Or alternatively, keep passaging them at 1:1 ratio until they are confluent enough to be transferred to a larger surface area.

6. Cell density of 8×10^3 cells per well of a 6-well plate works well for neonatal fibroblasts. For adult cells, density might need to be doubled for optimal results.
7. When adapting difficult lines to feeder-free conditions, try passaging back on MEFs with the addition of 10 μM Y-27632 for 1 day to enhance cell survival.

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Modeling Axonal Phenotypes with Human Pluripotent Stem Cells

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Abstract

Impaired axonal development and degeneration are implicated in many debilitating disorders, such as hereditary spastic paraplegia (HSP), amyotrophic lateral sclerosis (ALS), and periphery neuropathy. Human pluripotent stem cells (hPSCs) have provided researchers with an excellent resource for modeling human neuropathologic processes including axonal defects in vitro. There are a number of steps that are crucial when developing an hPSC-based model of a human disease, including generating induced pluripotent stem cells (iPSCs), differentiating those cells to affected cell types, and identifying disease-relevant phenotypes. Here, we describe these steps in detail, focusing on the neurodegenerative disorder HSP.

Keywords: hESCs, iPSCs, Axon, Mitochondria, Degeneration

1 Introduction

It became evident shortly after the first successful derivation of human embryonic stem cells (hESCs) (1) and iPSCs (2) that these cells would be invaluable for researchers looking to study human disorders, particularly diseases affecting the nervous system, where access to tissue is limited. Because these cells retain mutations present in patients, they can recapitulate many of the phenotypes in neurodegenerative disorders while maintaining the correct human genetic background. In addition, developmental biology has progressed at a rate that has allowed the directed differentiation of human pluripotent stem cells (hPSCs) to numerous distinct cellular populations, including various neuronal subtypes (3–12). These properties of hPSCs make them an ideal system to study neurodegenerative disorders in human cells.

In order to generate an accurate model of a neurodegenerative disorder, there are several important steps that must occur. The first step is to generate hPSCs that will be used to model a disease of interest. This can be accomplished in multiple ways, including generating iPSCs from affected patients and controls, or if a disease occurs through a haploinsufficiency mechanism, knockdown hESCs can be generated (*see* **Note 1**). The latter is useful

when access to patient tissue is limited or when confirming findings observed in patient-derived iPSCs. Next, the hPSCs are differentiated to the cell type that is affected in patients. In our case, telencephalic glutamatergic neurons are the main cell type which are affected in hereditary spastic paraplegia. Confirming that a sufficient percentage of the differentiated cells are the correct cell type and that there is not a dramatic difference between lines is crucial and must be determined prior to examining disease-specific phenotypes. Lastly, once the appropriate neurons are generated, you can examine the phenotype of the mutant cells compared to controls. Here, we will focus on two common defects observed in axonal degenerative diseases: neurite outgrowth deficits and alterations to fast axonal transport of mitochondria.

Impaired axonal functions underlie many neurodegenerative diseases, especially those which affect projection neurons. One process that is often affected is axonal transport. Projection neurons rely heavily on fast axonal transport to deliver membrane proteins, synaptic vesicles, axolemmal components, and mitochondria to the distal portions of the cell from the soma (13). A number of additional adult onset neurodegenerative diseases display defects in axonal transport, including amyotrophic lateral sclerosis (14), spinal muscular atrophy (15), Huntington's disease (16–18), and Alzheimer's disease (19–22). Mutations affecting the plus-end directed MT-based motor kinesin heavy chain (kinesin family, member 5A) result in a form of HSP (SPG10), confirming that perturbations to fast axonal transport can result in axonal degeneration (23). The fact that the majority of protein is generated within the soma and must be transported throughout the axon via anterograde transport makes efficient transport particularly critical in neurons (24). Transport of organelles, including mitochondria, is also essential for neurons (25, 26). Mitochondria move in a saltatory fashion; where some are rapidly transported long distances within axons, others appear stationary (27–31). Kinesin proteins mediate transport in the anterograde direction (32, 30, 33), while cytoplasmic dynein mediate transport in the retrograde direction (30, 34). The precise regulation of mitochondrial localization is necessary for regulating energy demand and cell survival (35). Here we will describe how to image and analyze mitochondrial transport in hPSC-derived neurons.

2 Materials

2.1 Stock Solutions

1. DMEM (1×): Life Technologies, cat. no. 11965-092.
2. DMEM/F12 (1×): Life Technologies, cat. no. 11330-032.
3. Fetal bovine serum (FBS): Life Technologies, cat. no. 16000-044.

4. Knockout Serum Replacement (KOSR): Life Technologies, cat. no. 10828-028.
5. Neurobasal Medium (1×): Life Technologies, cat. no. 21103-049.
6. N-2 (100×): Life Technologies, cat. no. 17502-048.
7. B27 (50×): Life Technologies, cat. no. 17504-044.
8. GlutaMAX (100×): Life Technologies, cat. no. 35050-061.
9. Nonessential amino acids (100×): Life Technologies, cat. no. 11140-050.
10. Trypsin-EDTA (0.05 %): Life Technologies, cat. no. 25300-054.
11. Phosphate buffered saline, pH 7.4 (1×): Life Technologies, cat. no. 10010-023.
12. StemPro Accutase (1×): Life Technologies, cat. no. A11105-01.
13. Trypsin inhibitor (1 mg/mL): Dissolve 50 mg in 50 mL of DMEM F/12, and then pass through a 0.22 μm Steriflip filter.
14. FGF2 (100 μg/mL): Dissolve 100 μg into 1 mL of sterile PBS with 0.1 % BSA (PeproTech, cat. no. 100-18B).
15. Ascorbic acid (200 μg/mL): Dissolve 2 mg ascorbic acid in 10 mL PBS. Store at −80 °C.
16. Cyclic AMP (1 mM): Dissolve 4.914 mg cyclic AMP in 10 mL sterilized water. Store at −80 °C.
17. BDNF, GDNF, IGF1 (100 μg/mL): Dissolve 100 μg of growth factor in 1 mL of sterile water.
18. Mouse Laminin (1 mg/mL): Invitrogen cat. no. 23017-015, aliquot and store at −80 °C until use. After thawing, store at 4 °C.
19. MitoTracker CMXRos (50 nM): Dilute 0.5 μL 1 mM stock into 10 mL of NDM medium (Life Technologies, cat. no. M-7512).

2.2 Media

1. MEF media (1 L): Filter 890 mL DMEM, 100 mL FBS, and 10 mL NEAA through a 0.22 μm filter flask.
2. hESC media (1 L): Filter 785 mL DMEM/F12, 200 mL KOSR, 10 mL NEAA, 5 mL GlutaMAX, and 7 μL β-ME through a 0.22 μm filter flask.
3. Neural induction media (NIM, 500 mL): Add 5 mL N2, 5 mL NEAA, and 1 mL of 1 mg/mL heparin to 489 mL of DMEM/F12. Add 1:50 B27, 1:1,000 cAMP, 1:10,000 IGF1 for neural differentiation.
4. Neural differentiation media (NDM, 50 mL): Add 0.5 mL N2, 0.5 mL B27, and 0.5 mL NEAA to 48.5 mL of Neurobasal Medium. Prior to use, add 1:1,000 cAMP, 1:1,000 ascorbic acid, 1:1,000 laminin, 1:10,000 IGF1, 1:10,000 BDNF, 1:10,000 GDNF.

2.3 Transfection Reagents

1. pCXLE-hOCT3/4-shp53-F: Addgene plasmid 27077.
2. pCXLE-hSK: Addgene plasmid 27078.
3. pCXLE-hUL: Addgene plasmid 27080.
4. pCXLE-EGFP: Addgene plasmid 27082.
5. NHDF Nucleofector Kit: Lonza, cat. no. VPD-1001.

2.4 Tissue Culture Supplies

1. 10 mL glass pipettes.
2. 6-well tissue culture-treated dishes.
3. T75 non-treated flask.
4. Glass bottom 35 cm dish: MatTek, cat. no. PG35G-1.5-14-C.

2.5 Antibodies

1. IgM anti-Tra-1-60: Santa Cruz, 1:50, cat. no. sc-21705.
2. Goat polyclonal IgG anti-Nanog: R&D Systems, 1:500, cat. no. AF1997.
3. Mouse monoclonal IgG3 anti-SSEA-4: Developmental Studies Hybridoma Bank, 1:100, cat. no. MC-813-70.
4. Rabbit IgG anti-Tau: Sigma-Aldrich, 1:100, cat. no. T6402.
5. Rabbit polyclonal anti-Tbr1: Proteintech, 1:1,000, cat. no. 20932-1-AP.
6. Mouse monoclonal IgG βIII-tubulin (TuJ-1): Abcam, 1:2,000, cat. no. ab18207.

2.6 Software

1. ImageJ (36).
2. ImageJ Plugin Straighten (37).
3. MetaMorph (Molecular Devices).

3 Methods

3.1 Generating iPSCs

The method for generating iPSC lines from human fibroblasts using episomal vectors is on the basis of the previous study from Dr. Yamanak's group (38).

1. Combine 1 μg of each of the four plasmids (pCXLE-hOCT3/4-shp53, pCXLE-hSK, pCXLE-hUL, pCXLE-EGFP) with 82 μL Human Dermal Fibroblast Nucleofector Solution and 18 μL supplement (from the Lonza NHDF Nucleofector Kit) for each line.
2. When fibroblasts are roughly 90 % confluent, trypsinize the cells, centrifuge 0.6×10^6 cells and remove medium, and resuspend in the Nucleofector solution from step 1.
3. Transfect the cells using a Nucleofector-2b with the program P-022.

4. Allow cells to recover for 10 min, and then plate them onto a 6-well TC-treated plate.
5. Replace MEF medium every 2 days.
6. On day 7, split cells onto a 10 cm dish with a mouse embryonic fibroblast (MEFs) feeder layer. Change medium to hESC medium +4 ng/mL bFGF.
7. Around day 21, iPSC colonies should be large enough to be split. Carefully remove individual colonies using a sterile syringe and a P200 pipette tip, and plate each in its own well of a 12-well dish on MEF feeders. If possible, try to break up the colony a little bit before plating to increase the number of colonies. Try to collect at least ten clones per line, most of which can be stored as backups. In general, two to three clones per line are enough to use for analysis, so the others can be stored in liquid nitrogen.
8. Expand the separate clones and split to a 6-well dish when they colonies become large enough.

3.2 Validating iPSC Lines

It is important to confirm that the generated iPSC lines are fully reprogrammed. A number of different methods can be employed to show this; however, we generally rely on staining for pluripotency markers such as Tra-1-60, SSEA4, and Nanog and the teratoma assay (39).

3.3 Differentiation to Affected Neuronal Subtypes

One of the main benefits of hPSCs is the ability to differentiate these cells to cell types which are affected in disease of interest, in our case telencephalic glutamatergic neurons. The steps below are summarized in Fig. 1a.

3.3.1 Days 0–4: Collecting hPSCs in Suspension

1. To generate telencephalic neurons from iPSCs, stem cells were cultured on a feeder layer of irradiated MEFs in 6-well tissue culture-treated plates for around 6 days, with the hESC media (+10 ng/mL fibroblast growth factor [FGF]-2) changed daily.
2. When nearly confluent, each well was washed once with 1 mL of PBS, and then 1 mL of Dispase was added. Cells were incubated for around 3 min at 37 °C or until the edges of colonies begin to round up.
3. The Dispase solution is removed and the wells are again washed with PBS, and then hESC media is added.
4. Next, scrape of the cells off of the well using a 10 mL glass pipette.
5. Transfer the cells to a nonadherent T75 flask with roughly 50 mL of hESC medium (adjust depending on amount of cells). Replace hESC medium every day.

3.3.2 Days 5–8: Neural Specification

6. Remove hESC medium and suspend cells in roughly 30 mL of NIM. Replace half of the media every other day.

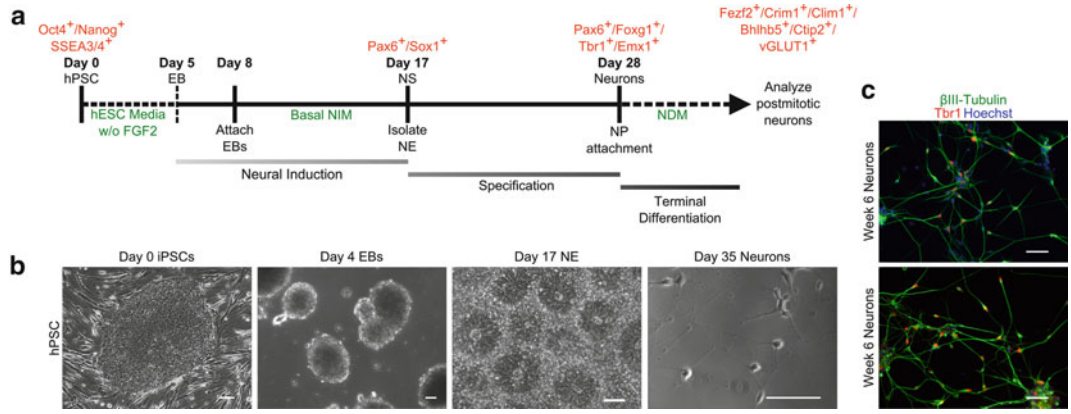

Fig. 1 Telencephalic glutamatergic neuron differentiation. (**a**) Schematic of the neural differentiation protocol used in this study. Cell markers are shown above and can be checked during the course of differentiation through immunocytochemistry. *EBs* embryoid bodies, *NE* neuroepithelium, *NP* neural progenitors. (**b**) Representative images of cells at various stages of differentiation. Scale bar: 100 μm. (**c**) Efficient generation of $Tbr1^+$ telencephalic glutamatergic neurons, stained at 6 weeks. Images in **b** and **c** have been adapted from our previous publication (47). Scale bar: 50 μm

3.3.3 Days 9–16: Formation of Neuroepithelial (NE) Cells

7. Next, the cells are plated onto 6-well dishes. Remove NIM and resuspend iPSC aggregates in enough NIM +5 % FBS so that 1.5 mL can be used for each well of a 6-well plate. Allow the aggregates to attach overnight, and in the next morning, remove the media and replace with standard NIM. Replace media every other day until day 17. During this time, NE cells will develop, and form characteristic "rosette" structures, which resemble the neural tube (Fig. 1b).
8. It is also recommended to test the neural induction efficiency around day 10. To do this, staining for $Pax6^+$ cells is recommended (*see* **Note 2**).

3.3.4 Day 17–27: Specification of Telencephalic Neurons

9. On day 17, remove the NE cells by gently blowing media onto the colonies using a P1000 pipette. Collect the isolated NE cells and culture in suspension with NIM supplemented with 1:50 B27, 1:1,000 cAMP, and 1:10,000 IGF1.
10. Replace half of the media every other day until day 27. Once a week, the neurospheres can be fragmented into smaller clusters by passing them through a P200 pipette several times.

3.3.5 Day 28: Plating Neurons

11. Wash neurospheres with PBS one time, and incubate in Accutase for 2 min at 37 °C.
12. Add an equal volume of trypsin inhibitor solution, and then centrifuge the cells for 2 min at 400 × *g*.
13. Remove the media and resuspend the neurospheres in 50 μL neural differentiation media (NDM). Pipette up and down

several times with a P200 tip to dissociate the cells. Add additional NDM medium so there is a sufficient volume to reduce the density of cells.

14. Add the cells to poly-ornithine-/laminin-coated coverslips and incubate cells for at least 2 h at 37 °C so that they have time to attach.
15. Once attached, slowly add 500 μL of NDM to prevent cells from detaching. Replace 250 μL of NDM medium every other day until the cells are ready for analysis.

3.4 Confirming the Generation of Telencephalic Glutamatergic Neurons

We perform immunostaining of the generated neurons around 6 weeks after differentiation began to determine the percentage of telencephalic glutamatergic neurons that were generated using the markers that are listed in Fig. 1a. In Fig. 1c, $Tbr1^+$ and $\beta III\text{-}tubulin^+$ cells are shown at 6 weeks. This step confirms that there is not a dramatic difference between control and mutant groups at generating these cells (*see* **Note 3**).

3.5 Identifying Disease-Related Phenotypes

Once you are confident that the appropriate neuronal subtype has been generated (*see* **Note 4**), you can next begin to identify disease-related phenotypes in your mutant cells. We chose to look for axonal defects since HSP is associated with distal axonal degeneration (40). The two methods that we will discuss in detail are measuring neurite outgrowth and fast axonal transport of mitochondria.

3.5.1 Measuring Neurite Outgrowth

In order to look at initial neurite outgrowth, cells are analyzed just 48 h following plating onto coverslips; representative images are shown in Fig. 2a. This time point was also chosen because the coverslips will become too dense with neurites after longer periods of time, making it difficult to trace the morphology of individual cells.

1. Plate dissociated neurons at a low density, and allow neurites to extend for 48 h (*see* **Note 5**).
2. Fix cells with 4 % paraformaldehyde and stain cells for Tau and MAP2 to stain axons or dendrites respectively.
3. Image the cells using a 20× objective and quantify neurite outgrowth using the NeuronJ plugin (41) for ImageJ.
4. For each neuron, trace primary and secondary neurites. Axons can be identified as the longest neurite which also stains the strongest for Tau.

3.5.2 Analyzing Mitochondrial Transport

1. Plate dissociated neurons onto 35 cm glass-bottom dishes (Matek) following the steps as above.
2. When the cells have matured sufficiently, stain the live cells with 50 nM MitoTracker CMXRos for 3 min at 37 °C.
3. Wash the cells two times with NDM.

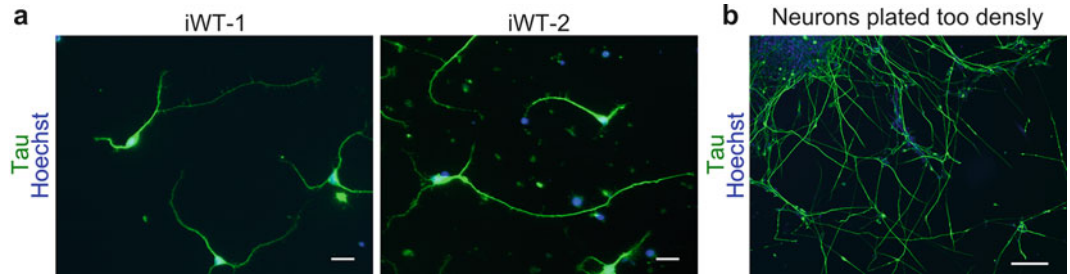

Fig. 2 Neurons 48 h post plating stained for Tau. (**a**) These cells were plated at a good density, making it easy to trace neurites extending from each cell. Scale bar: 20 μm. (**b**) These cells were not adequately dissociated and were cultured for 2 weeks after plating, causing the neurites to become too dense. Accurate tracing of neurites would be difficult with this image. Scale bar: 100 μm

4. Image cells using a 63× objected with a microscope that has a heated stage (37 °C).
5. Visually identify axons based on morphological characteristics (constant thin diameter, long neurites, no branching, and direct emergence from the cell body (42)).
6. After identifying a suitable region, set up a time series by taking an image every 5 s for at least 5 min (*see* **Note 6**).
7. Save the images as TIFs, and open all images within one series in ImageJ. Combine all images into a TIF stack (Image → Stacks → Images to Stack)
8. Next, use the ImageJ plugin "Straighten" to straighten imaged axons (Fig. 3a) (37). Be sure to start with the proximal portion of the axon, as this will appear on the left side of the generated image series, making it easier to calculate directional transport parameters. Adjust filament/wide line value so that the width of the neurite is completely covered.
9. Open the straightened TIF series in MetaMorph and calibrate the scaling for the images.
10. Open the Track Points app (Apps → Track Points) and first set the origin (Set Origin → Click on the far left side, towards the cell body → OK). Also set the interval to 5 s (Set Interval → User defined → 5 seconds → OK) and configure set the program to record all parameters (Config Log → Enable All).
11. Manually track the location of one mitochondrion within the axon for every frame in the series, and then "Add Track" for the next mitochondrion. Repeat until the location of every mitochondrion within the axon has been tracked.
12. Open Log.
13. Dynamic Data Exchange (opens Excel) → OK (keep default options).
14. F9: Log Data.

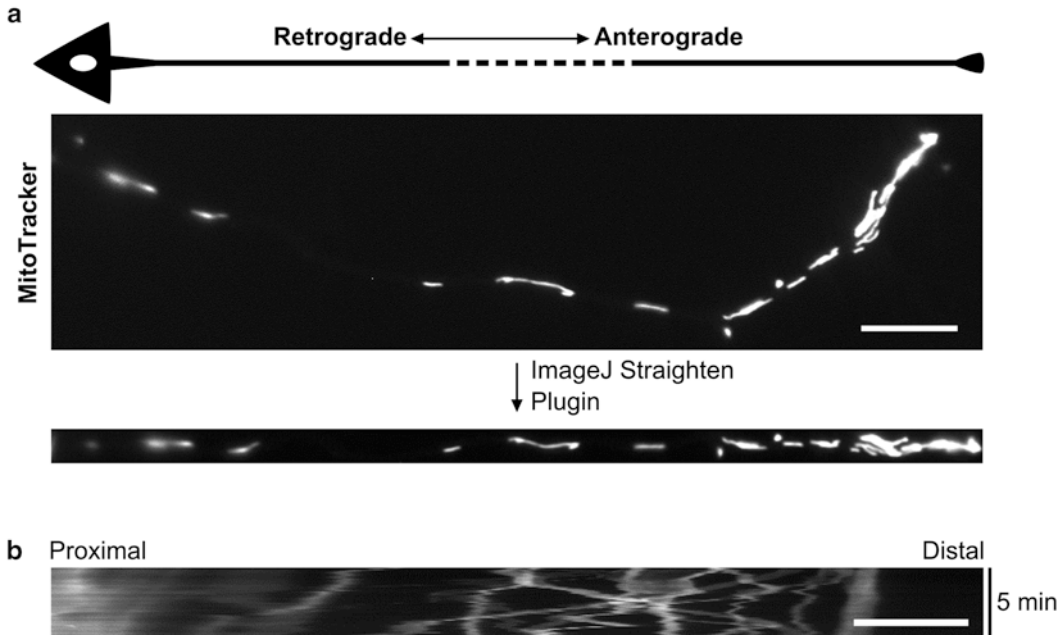

Fig. 3 Analyzing mitochondrial transport. (**a**) Mitochondria within an axon imaged after staining with MitoTracker CMXRos. The cell soma was located to the *left* of the field. *Bottom image* shows results of the Straighten plugin in ImageJ. (**b**) Representative distance versus time kymograph generated in Metamorph. Scale bar: 10 μm

15. Enter all of the Track Point data into Excel, grouped by each Track # (mitochondrion).
16. Decide what parameters you are interested in: mean velocity, anterograde/retrograde velocity, motile events, anterograde/retrograde events, average run velocity, percentage motile mitochondria per cell, pause/run lengths.
17. Manually calculate velocity with the equation below, since MetaMorph does not differentiate between anterograde and retrograde transport.

$$= \frac{(\text{Distance from origin at current time} + \text{distance at previous time point})}{5\,s}$$

    (a) Positive values = anterograde transport
    (b) Negative value = retrograde transport
18. Determine motility events using a $\geq \pm 300$ nm/s threshold, which excludes events that may be mediated by actin-based transport and should only include microtubule-based transport (42).
19. If there are three consecutive motile events in one mitochondrion's time series, then that mitochondrion is considered motile.

20. Organize group data and compare control to mutant lines.
21. A distance versus time kymograph can be generated in MetaMorph for graphical representation of mitochondrial transport (Fig. 3b).

4 Notes

1. Depending on the type of mutation that is present in your iPSCs, it is often useful to generate hESC lines where a gene of interest is knocked down. These knockdown lines can be used to confirm that any phenotypes observed in mutant iPSC-derived cells are caused by haploinsufficiency and are not due to clonal variation. We design shRNAs using http://sirna.wi.mit.edu/ (43).
2. To test the efficiency of neural induction, flow cytometry can be used to compare the percentages of $Pax6^+$ cells between groups (44).
3. It is also recommended to stain and quantify the number of astrocytes ($GFAP^+$) and oligodendrocytes ($O4^+$) that are within neural cultures. This will ensure there are not dramatically different proportions of these cells between control and experimental groups.
4. Depending on your disease of interest, a number of different neuronal subtypes can be generated besides telencephalic glutamatergic neurons. These include spinal motor neurons (6) and dopaminergic neurons (45, 46). It is often useful to compare affected neuronal subtypes to a subtype that is not affected in a particular disease, to show cell type-specific phenotypes in your system.
5. Be sure not to plate neurons too densely for measuring neurite outgrowth, or it will be difficult to trace individual neurites (Fig. 2b).
6. When imaging mitochondrial movement, take care to adjust the exposure time and light intensity to prevent photobleaching.

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Mitochondrial Disease-Specific Induced Pluripotent Stem Cell Models: Generation and Characterization

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Abstract

Mitochondrial disease is a group of disorders caused by dysfunctional mitochondria, of which the mutation in the mitochondrial DNA is one of the primary factors. However, the molecular pathogenesis of mitochondrial diseases remains poorly understood due to lack of cell models. Patient-specific induced pluripotent stem cells (iPS cells or iPSCs) are originated from individuals suffering different diseases but carrying unchanged disease causing gene. Therefore, patient-specific iPS cells can be used as excellent cell models to elucidate the mechanisms underlying mitochondrial diseases. Here we present a detailed protocol for generating iPS cells from urine cells and fibroblasts for instance, as well as a series of characterizations.

Keywords: Induced pluripotent stem cells, Urine cells, Fibroblasts, Characterization

1 Introduction

Mitochondrial disease is a group of disorders caused by dysfunctional mitochondria, in which the mutation in the mitochondrial DNA (mtDNA) is one of the primary factors (1). However, the molecular pathogenesis of mitochondrial diseases remains poorly understood due to lack of cell and animal models. On the one hand, it is limited to obtain the experimental cells excepting from blood, urine, hair and skin. The functional assays of these cells could not provide direct evidence for the impact of mtDNA mutation on those pathogenic cells. On the other hand, no high-performance plasmid systems yet to modify mtDNA or make particular mtDNA mutation, because each mammalian cell contains hundreds of double membrane-bound mitochondria, and each mitochondrion contains multiple copies of mtDNA molecules (2, 3). Induced pluripotent stem cells (iPS cells or iPSCs) are a type of pluripotent stem cells artificially derived from adult somatic cells. iPS cells were first generated by Yamanaka in 2006 through enforced expression of four transcription factors (Oct3, Sox2, Klf4, and c-Myc) with retrovirus vector (4). Patient-specific iPS cells are originated from individuals suffering different diseases but carrying unchanged

disease causing gene. Therefore, patient-specific induced pluripotent stem cells and differentiated cells derived from them are attracting increasing attention to elucidate the mechanisms underlying mtDNA diseases.

The overwhelming ascendancy of patient-specific iPS cells is that the sources can be any individual patient somatic cells. So far, many cell types have been switched to iPS cells, including fibroblast (5), peripheral blood cells (6), periosteal membrane (7), umbilical cord matrix, amniotic membrane mesenchymal cells (8), and exfoliated renal tubular cells (9). Acquiring these types of cells is low traumatic and done with few contraindications to donors, and they can provide numerous cell materials for disease research.

iPS cells can be directly differentiated into specific tissue. With the differentiation potential, patient-specific iPS cells will permit the production of unlimited numbers of specific cell types needed to model disease. It would seem preferable to illustrate how the gene mutation leads to disorder from studies with the specific diseased cells. In comparison to many general cellular models, such as those using fibroblasts or lymphocytes, differentiated derivatives from iPS cells provide a much more direct indication of the process of how the mutation results in disease.

Patient-specific iPS cells thus offer an attractive window into understanding disease development. Their in vitro culture system can provide a rapid and cost-effective way to interrogate the function of a gene during a specific developmental process. Patient-specific iPS cells are therefore highly relevant in the study of multiple stages of pathogenesis, from the initial cellular abnormality to the hierarchical organization of established dysfunction or even cell death, providing a human cell model to study the stages of disease (10). Thus, it will help us to investigate disease mechanisms to a greater depth than was previously feasible.

Since the first generation of iPS cells, they have led to a new situation in the field of stem cell research and been experiencing years of development. Refinements in reprogramming techniques have also been achieved recently (11). These include various nonviral methods such as DNA plasmid transfection or modified mRNA (12, 13) and nonintegration adenoviral vectors (14, 15), together with less than four transcription factors (16, 17). For the most part, these methods have reprogramming efficiencies of approximately 0.1–1 % of that described for retroviral transduction and they are also technically challenging (18). Because patient-specific iPS cells are extended from general iPS cells, their generation and characterization are coincident. Here we present a detailed protocol for generating patient-specific iPS cells from fibroblasts and urine cells in particular. The induction process contains two stages, isolation of primary cells and generation of iPS cells. Characterization includes alkaline phosphatase, immunofluorescence, real-time PCR, bisulfite genomic sequencing, semiquantitative PCR and G-banding

chromosome analysis, embryoid body induction and teratoma formation. Only via such a series of characterizations are these generated iPS cells confirmed to show the properties of embryoid stem (ES) cells, and are affirmed to be induced pluripotent stem cells.

2 Materials

2.1 Reagents for Fibroblast and Urine Sample Collection

1. Fibroblast medium: Fibroblast medium contains DMEM/high glucose with 10 % (vol/vol) FBS, 1 % (vol/vol) NEAA, 1 % (vol/vol) L-GlutaMAX. Store at 4 °C.
2. Primary urine cell medium: REGM™ Renal Epithelial Cell Growth Medium BulletKit® and Fibroblast medium as the proportion of 1:1. Store in a dark place, at 4 °C.
3. General store solution: 90 % (vol/vol) FBS (PAA), 10 % (vol/vol) DMSO. Prepare immediately prior to use.
4. Other reagents: Penicillin/Streptomycin, Primocin, 0.1 % Gelatin, MycoAlert® Mycoplasma Detection Kit.
5. Equipment: Biological safety cabinet, CO_2 incubator, tissue culture dishes (60 mm/100 mm), tissue culture plates (6/12/24 wells), centrifuge tubes (15 and 50 mL).

2.2 Reagents for Retrovirus Production and iPS Cells Induction

1. Plasmids: Four transcription factors: pMXs-hSOX2, pMXs-hOCT4, pMXs-hKLF4, pMXs-hc-MYC, pMXs-GFP, and pCL-Eco.
2. Reagents for calcium phosphate transfection:
   2× HBS: 280 mM NaCl, 10 mM KCL, 1.5 mM Na_2HPO_4, 50 mM Hepes, 12 mM glucose, Sterilization by filtration with a 0.22-μM syringe filter, adjustment of pH value to 6.95–7.05.
   2 M $CaCl_2$: Sterilization by filtration with a 0.22-μM syringe filter.
3. 1,000× bFGF(8 μg/mL): Reconstitute recombinant human fibroblast growth factor-basic 10 μg in 1.25 mL DPBS with 0.1 % (wt/vol) BSA. Filter-sterilization with a 0.22-μm syringe filter. Store at −20 °C.
4. Human ESC medium: Human ESC medium contains DMEM/F12 supplemented with 20 % (vol/vol) Knockout serum replacement (KSR), 1 % (vol/vol) GlutaMAX, 1 % (vol/vol) NEAA, 100 μM β-mercaptoethanol, 1× (8 ng/mL) bFGF. Store at 4 °C.
5. Human dFBS medium: DMEM/HG containing 20 % (vol/vol) defined FBS (dFBS) 1 % (vol/vol) GlutaMAX, 1 % (vol/vol) NEAA, 100 μM β-mercaptoethanol, 8 ng/mL bFGF. Store at 4 °C.
6. Human ESC-dFBS medium: mix human ESC and human dFBS medium in at a 1:1 ratio. Store at 4 °C.

7. hPSC store solution: hPSC store solution contains 30 % dFBS, 60 % human ESC medium and 10 % DMSO. Prepare immediately prior to use.
8. mTeSR1: add the mTeSR1 5× supplement into the mTeSR1 basal medium. Store at 4 °C.
9. 10× dispase: Dissolve 0.1 mg dispase in 10 mL DMEM/F12. Filter-sterilize with a 0.22-μm syringe filter. Store at 4 °C.
10. 1,000× Vc (50 mg/mL): Dissolve 0.5 g ascorbic acid (Vc) in 10 mL H_2O. Filter-sterilize with a 0.22-μm syringe filter. Store at −20 °C.
11. VPA (200 mM): Resuspend VPA in DMEM/F12 to a final concentration of 200 Mm. Filter-sterilize with a 0.22-μm syringe filter. Store at −20 °C.
12. EDTA (50 mM): Resuspend EDTA.2Na.2H_2O in DMEM/F12 to a final concentration of 50 mM. Filter-sterilize with a 0.22-μm syringe filter. Store at 4 °C.
13. Matrigel: Thaw Matrigel slowly on ice. Pipet 0.25 mL in per prechilled 1.5 mL tubes and tore them at −80 °C. Before use, thaw a 0.25 mL aliquot on ice and dissolve it in 25 mL cold DMEM/F12. Store at 4 °C.
14. Equipment: Disposable Pasteur pipettes, 0.45/0.22 μM syringe filter.

2.3 Reagents for Characterization of iPS Cell

1. Alkaline Phosphatase Staining Kit.
2. Reagents needed to be prepared: PBS; 4 % paraformaldehyde (4 % PFA); 1× TBST: 20 mM Tris–HCl (pH 7.4), 0.15 M NaCl, 0.05 % Tween 20.

2.3.1 Alkaline Phosphatase Staining

2.3.2 Immunofluorescence Microscopy

1. Blocking solution: PBS containing 3 % (wt/vol) BSA and 0.2 % Triton X-100.
2. Primary antibodies: SSEA3 (1:100); SSEA4 (1:100); Nanog (1:150); TRA-1-60 (1:100); TRA-1-81 (1:100).
3. Second antibodies: Alexa Fluor 594-conjugated goat anti-rat IgM (1:1,000); Alexa Fluor 594-conjugated donkey anti-mouse IgG (1:1,000); Alexa Fluor 594-conjugated donkey anti-goat IgG (1:1,000); Alexa Fluor 488-conjugated goat anti-mouse IgM (1:1,000).
4. Other reagents and equipment: DAPI, Pasteur pipette, sealing film.

2.3.3 RT-PCR for Marker Genes

1. Reagents: TRIzol.
2. Equipment: 1.5 mL RNase-free microcentrifuge tubes and RNase-free pipette tips.

3. Kit: TRIzol Plus RNA Purification Kit, PrimeScript RT Reagent Kit, SYBR Green Premix EX Taq™, Thermal Cycler Dice™ Real Time System.
4. Primer list for exogenous reprogramming of genes.

Gene	Primer (5′ → 3′)	
Tg-hOct4	Forward	GGGTGGACCATCCTCTAGAC
	Reverse	CCAGGTCCGAGGATCAAC
Tg-hSox2	Forward	GGGTGGACCATCCTCTAGAC
	Reverse	GGGCTGTTTTTCTGGTTG
Tg-hKlf4	Forward	GGGTGGACCATCCTCTAGAC
	Reverse	GGAAGTCGCTTCATGTGG
Tg-hc-Myc	Forward	GGGTGGACCATCCTCTAGAC
	Reverse	CCTCGTCGCAGTAGAAATAC

5. Primer list for endogenous pluripotent genes.

Gene	Primer (5′ → 3′)	
HS ACTB	Forward	CCCAGAGCAAGAGAGG
	Reverse	GTCCAGACGCAGGATG
HS endoOct4	Forward	CCTCACTTCACTGCACTGTA
	Reverse	CAGGTTTTCTTTCCCTAGCT
HS endoSox2	Forward	CCCAGCAGACTTCACATGT
	Reverse	CCTCCCATTTCCCTCGTTTT
HS endoNanog	Forward	TGAACCTCAGCTACAAACAG
	Reverse	TGGTGGTAGGAAGAGTAAAG
HS endoRex1	Forward	TCGCTGAGCTGAAACAAATG
	Reverse	CCCTTCTTGAAGGTTTACAC

2.3.4 Integration of the Exogenous Transgenes by Semiquantitative PCR

1. TaKaRa Universal Genomic DNA Extraction Kit.
2. Primer list for integration of the exogenous transgenes.

Gene	Primer (5′ → 3′)	
Inte-hOct4	Forward	GAGAACCGAGTGAGAGGCAAC
	Reverse	TTATCGTCGACCACTGTGCTG
Inte-hSox2	Forward	CTTGGCTCCATGGGTTCG
	Reverse	TTATCGTCGACCACTGTGCTG
Inte-hKlf4	Forward	TCTCTTCGTGCACCCACTTG
	Reverse	TTATCGTCGACCACTGTGCTG
Inte-hc-Myc	Forward	AGAGTCTGGATCACCTTCTGCTG
	Reverse	TTATCGTCGACCACTGTGCTG

2.3.5 Karyotype Analysis

1. Materials requiring preparation: Hypotonic solution containing 0.56 % (g/mL) potassium chloride and 0.5 % (g/mL) sodium citrate. Fixing solution contained methyl alcohol and ethanoic acid in at a proportion of 3:1. 0.1 % trypsin was prepared in 0.8 % sodium chloride.

2.3.6 Bisulfite Sequencing Analysis

1. TaKaRa Universal Genomic DNA Extraction Kit.
2. EcoRV, mineral oil, hydroquinone, sodium metabisulfite, low gelling temperature agarose, Taq, pMD19-T Simple Vector.
3. Materials requiring preparation. Sodium metabisulfite solution (prepared in darkness): add 0.44 g hydroquinone in 4 mL ddH_2O and incubate at 50 °C until totally dissolved. 15.2 g sodium metabisulfite is then dissolved in 16 mL ddH_2O, 10 mL 2M NaOH is added and then mixed by flicking the tube up and down to ensure they are completely dissolved. Pipet the above hydroquinone solution into the dissolved sodium metabisulfite with sufficient mixing and incubate at 50 °C for 15–20 min. Store at 4 °C. Before use, incubate the solution at 50 °C to dissolve the crystalline solid.
4. Amplified regions of Oct4 gene promoter.

Site	Region
−204	ttccacagac accattgcca ccaccattag gcaaacatcc ttcgcctcag tttctccccc
−144	cacctccctc tcctecaccc atccaggggg cggggccaga ggtcaaggct agtgggtggg
−84	actggggagg gagagagggg ttgagtagtc ccttcgcaag ccctcatttc accaggcccc
−24	cggcttgggg cgccttcctt ccccatggcg ggacacctgg cttcggattt cgccttctcg
37	ccccctccag gtggtggagg tgatgggcca gggggggccgg agccgggctg ggttgatcct
97	cggacctggc taagcttcca aggccctcct ggagggccag gaatcgggcc gggggttggg
157	ccaggctctg aggtgtgggg gattccccca tgccccccgc cgtatgagtt ctgtgggggg

5. Amplified regions of Nanog gene promoter.

Site	Region
−624	agagaggggg tttcgccatg ttggccaggc tggtttcaaa ctcctgactt caggtgatcc
−564	gcctgccacg gcctcccaat ttactgggat tacaggggtg ggccaccgcg cccggccttt
−504	ttcttaattt ttaaaaatat taaagtttta tcccattcct grtgaaccat attcctgatt
−444	taaaagttgg aaacgtggtg aacctagaag tatttgttgc tgggtttgtc ttcaggttct
−384	gttgctcggt tttctagttc cccacctagt ctgggttact ctgcagctac ttttgcatta
−324	caatggcctt ggtgagactg gtagacggga ttaactgaga attcacaagg gtgggtcagt

6. Primer lists for first round nested-PCR.

Genes	Primer (5′ → 3′)	
Hs-Nanog-meth	Forward	TGGTTAGGTTGGTTTTAAATTTTT
	Reverse	AACCCACCCTTATAAATTCTCAATTA
HsOct4-meth	Forward	TTATTGTTATTATTATTAGGTAAATATTT
	Reverse	AAAATCCCCCACACCTCAAAACCTAACC

7. Primer lists for second round nested-PCR.

Genes	Primer (5′ → 3′)	
Hs-Nanog-meth	Forward	TTAATTTATTGGGATTATAGGGGTA
	Reverse	AACAACAAAACCTAAAAACAAACC
HsOct4-meth	Forward	GGGGTTAGAGGTTAAGGTTAGTAGGGTA
	Reverse	AAACCTTAAAAACTTAACCAAATC

2.3.7 In Vitro Differentiation

1. EB medium: EB medium contains DMEM/F12 supplemented with 20 % (vol/vol) Knockout serum replacement, 1 % (vol/vol) GlutaMAX, 1 % (vol/vol) NEAA, 100 μM β-mercaptoethanol. Store at 4 °C.
2. Collagenase IV: 10 mg in 10 mL DMEM/F12. Filter-sterilize with a 0.22-μm syringe filter. Store at −4 °C.
3. Primer list.

Genes	Primers (5′ → 3′)	
HS AFP	Forward	ATTGGCAAAGCGAAGCTG
	Reverse	GCTGTGGCTGCCATTTTT
HS GATA4	Forward	CAGAAAACGGAAGCCCAA
	Reverse	TTGCTGGAGTTGCTGGAAG
HS SOX17	Forward	ACGGAATTTGAACAGTAT
	Reverse	CAGGATAGTTGCAGTAAT
HS TBX1	Forward	AGCGAGAAATATGCCGAGG
	Reverse	TTCGCGAAGGGATTGCT
HS PAX6	Forward	TTGCTTGGGAAATCCGAG
	Reverse	TGCCCGTTCAACATCCTT
HS SOX1	Forward	TTTCCCCTCGCTTTCTCA
	Reverse	TGCAGGCTGAATTCGGTT
HS-Oct4	Forward	CCTCACTTCACTGCACTGTA
	Reverse	CAGGTTTTCTTTCCCTAGCT
HS-Nanog	Forward	TGAACCTCAGCTACAAACAG
	Reverse	TGGTGGTAGGAAGAGTAAAG

2.3.8 Teratoma Formation

1. 10× dispase: 0.1 mg dispase in 10 mL DMEM/F12. Filter-sterilize with a 0.22-μm syringe filter. Store at −4 °C.
2. Feeder-free iPS cell medium: mTeSR1, use with Matrigel as basement.

3 Methods

3.1 Primary Cells Preparation

Preparation of human dermal fibroblasts needs about 4 weeks. This part refers to ref. (19).

3.1.1 Isolation of Human Dermal Fibroblasts

1. Obtain a 6-mm skin punch biopsy and place in fibroblast medium immediately.
2. Mince the skin biopsy with sterile scissors.
3. Place the pieces in a well of a 6-well plate with 1 mL fibroblast medium.
4. Lower a coverslip onto the pieces and add fibroblast medium to a total 3 mL. Incubate at 37 °C, 5 % CO_2.
5. After 7–10 days, fibroblasts should appear. Aspirate the medium and wash with PBS twice. Lift the coverslip with forceps to ensure the medium had been washed away with PBS. Fibroblasts should remain attached to the coverslip.
6. Added 500 μL 0.05 % trypsin, and ensure the trypsin could overspread the coverslip with fibroblasts adhered. Digest at 37 °C for 5 min.
7. Flip the coverslip over with forceps to incubate for another 5 min.
8. Add inefficient fibroblast medium to stop reaction and disperse cells with a pipette. Collect cell suspension into a 15 mL tube.
9. Wash the well and coverslip with 3–5 mL fibroblast medium and transfer into the same tube.
10. Pass the cells through a 70-μM cell trainer. Centrifuge at 1,000 rpm for 5 min at room temperature.
11. Discarded the supernatant. Resuspend the cells with 5 mL fibroblast medium and plate into T-25 tissue culture flask.
12. When cells reach the density of 80 % confluency, passaged at a ratio of 1:3 on a 15 cm dish.
13. Cells can be split up to passage 10. If desired, freeze cells as above.

3.1.2 Isolation of Urinary Cells

Preparation of human urine cells needs about 2 weeks. This part refers to ref. (20).

1. Before urine collection, prepare two sterilized containers (up to 250 mL) for each donor labelling name, age and sex.

2. Add 5 mL penicillin/streptomycin (*see* **Note 1**) in each container. Store at −20 °C.
3. It is better to discard the first portion of the urine stream and collect the mid stream of urine into containers. If the urine output is abundant, it can be collected in two containers.
4. Transfer the urine samples into 50 mL tubes (*see* **Note 2**). Centrifuge at 400 × *g* for 10 min at room temperature.
5. Carefully discard the supernatant, leaving approximately 5 mL urine in the tube.
6. Resuspend the pellets and collect multiple tubes of urine which are from the same sample into one single 50 mL tube.
7. Add PBS containing penicillin/streptomycin up to 50 mL. Centrifuge at 400 × *g* for 10 min at room temperature.
8. Carefully aspirate the supernatant, leaving approximately 3 mL of the sample.
9. Add PBS containing penicillin/streptomycin up to 25 mL. Centrifuge at 400 × *g* for 10 min at room temperature.
10. Carefully discard the supernatant, leaving only around 0.5 mL of sample.
11. Add 1 mL of primary urine cell medium to resuspend the cell pellet and transfer the cells onto 6-well plate (coated with 0.1 % gelatin beforehand). Then add medium up to 3 mL (*see* **Note 3**).
12. Add 0.1 % primocin (3 μL). Incubate the cells in 37 °C, 5 % CO_2 incubator for 3 days.
13. Observe whether small colonies are present. If there are colonies, change 2 mL fresh medium. If there is not, add 2 mL medium and maintain incubation for another 2 days.
14. With the colonies growing, change fresh medium every 2 days. Observe the cells under microscope daily. These cells are labeled as Passage 0 (P0).
15. When cell density reaches 80–90 % confluency, they can be transferred onto 6 cm plate for further expansion. Aspirate the medium and wash the cells with PBS. Add 0.5 mL 0.25 % trypsin to digest for 2 min. Use fibroblast medium to stop reaction and blow off the colonies with a pipette. Then transfer the cell suspension into a 15 mL tube. Centrifuge the mixture at 1,000 rpm for 5 min at room temperature. Discard the supernatant and resuspend with 1 mL primary urinary medium. Transfer the cells into a 6 cm dish and add medium up to 4 mL. Incubate them in 37 °C, 5 % CO_2 incubator for 48 h. This is considered Passage 1 (P1).

16. When the culture reaches 80–90 % cell density, freeze P1 cells. Aspirate the medium and wash the cells with PBS. Add 0.5 mL 0.25 % trypsin to digest for 2 min. Use fibroblast medium to stop reaction and blow off the colonies with a pipette. Then transfer the cell suspension into a 15 mL tube. Centrifuge the mixture at 1,000 rpm for 5 min at room temperature. Discard the supernatant and resuspend with 1 mL general store solution, and count the cells using a hemocytometer or an automated cell counter. Store 6–8 $\times$ 10^5 in 1 mL of general store solution in one cryovial. Label the cell name, number, time, and operator. Put the cryovials in a freezing container at −80 °C overnight. Transfer freezing tubes from −80 °C to liquid nitrogen for later infection use (*see* **Note 4**).

3.1.3 Isolation of Other Human Differentiated Cells

1. Establishment of primary cultures of bone marrow mesenchymal cells (21).
2. Establishment of primary cultures of peripheral blood cells (6, 22, 23).

3.2 Generation of Induced Pluripotent Stem Cells from Urine Cells

Generation of iPS cells needs about 1 months. This part refers to ref. (9, 20).

1. Thaw a vial of HEK 293T cells in a 37 °C water bath. Wipe the vial with 75 % ethanol. In biological safety cabinet, transfer the HEK 293T cells into a 15 mL centrifuge tube. Carefully add a dropwise fibroblast medium of up to 5–6 mL. Centrifuge at 1,000 rpm for 10 min at room temperature. Discard supernatant, add 1 mL fibroblast medium to resuspend, and transfer cells onto a 10 cm dish.
2. After 1 or 2 days, cell density will reach about 90 % confluency. Passage the cells according to the proportion of 1:2 (with the method as step 15 in Section 3.1).
3. When the cells reach about 90 % confluency, aspirate the medium and wash with PBS once. Add 1 mL of 0.25 % trypsin. Digest for 2 min at room temperature. Stop reaction by adding 1 mL medium. Rinse the dish with 1–2 mL medium and collect the cell suspension into a 15 mL tube. Centrifuge at 1,000 rpm for 5 min at room temperature. Aspirate the supernatant and resuspend in 1 mL of medium and count the cells.
4. Seed 4–5 $\times$ 10^6 cells in 10 mL of fibroblast medium in every five 10 cm dish (*see* **Note 5**). Incubate them in 37 °C, 5 % CO_2 incubator. This is considered Day 1.
5. On Day 2, plasmid transfect HEK 293T cells for virus package. Here, we adopt calcium phosphate transfection technique. For a 10 cm dish, use 20 μg of every pMXs vector corresponding to 20 μg of pCL-Eco packaging plasmid. Before transfection,

change 5 mL fresh fibroblast medium for HEK 293T cells. Prepare five 15 mL tubes for the five plasmid (Oct4, Sox2, Klf4, c-Myc, and GFP control). Prepare the mixes in each 15 mL tube, in the following order: *X* mL H_2O, 20 μg of pCL-Eco, 20 μg of pMXs vector, 156.25 μL 2 M $CaCl_2$, [*X* mL H_2O = 2.5 mL − (volume of 20 μg of pMXs vector) − (volume of 20 μg of pCL-Eco) − (156.25 μL 2 M $CaCl_2$) − (1.25 mL 2× HBS)], mix them. Add 1.25 mL 2× HBS, pipetting vigorously to mix. Stand for 2 min (*see* **Note 6**). Add 2.5 mL fresh fibroblast medium to stop reaction. Mix them and gently add the mix dropwise and uniformly onto the HEK 293T cells. Incubate in 37 °C, 5 % CO_2 incubator overnight.

6. On Day 3, at 12–14 h transfection, carefully remove the medium and add 8.5 mL fresh medium in each 10 cm dish. Prepare two wells of urine cells at P2-P4 in a 6-well plate, 5–10 × 10^4 cells per well (*see* **Note 7**).
7. On Day 4, at 48 h post transfection, check the fluorescence produced by pMXs-GFP and make sure that nearly 90 % or above of HEK 293T cells have been transfected (*see* **Note 8**). Collect the viral supernatant of four transcription factors into a 50 mL tube. Additionally, collect the GFP viral supernatant into a 15 mL tube. Complement 10 mL of fresh fibroblast medium to each HEK 293T cell dish (*see* **Note 9**). Filter the four-factor and GFP viral supernatants with a 0.45 μM syringe filter. Add 8 mL of the four-factor viral supernatant onto one well of urine cells and add the 2 mL GFP viral supernatant onto another. Add polybrene to a final concentration of 8 μg/mL to each well. Incubate the cells in 37 °C, 5 % CO_2 incubator overnight.
8. On Day 5, 12–14 h after infection, aspirate the viral supernatant and add 2 mL of fresh primary urinary medium in each well. Collect the 72 h viral supernatant after transfection, as in the method in step 7, and discard the HEK 293T cells.
9. On Day 6, at 12–14 h after the second infection, change fresh primary urinary medium. Check the infection efficiency by observing the GFP virus expression on a fluorescence microscope. Only if most of the urine cells are infected by four exogenous factors, would they reprogram and form iPS cells.
10. On Day 7, continue to replace the medium with fresh primary urinary medium daily. The urine cells may become packed and some of them appear with an obvious nuclear–cytoplasmic ratio.
11. On Day 8–9, if the morphology change is substantial where the cells are more tightly packed with a more distinct nuclear–cytoplasmic ratio, transfer them into 10 cm dishes (normally on days 3–5 after first round infection). Aspirate off the medium in

6-well plate and wash once with PBS. Add 0.5 mL 0.25 % trypsin to digest at 37 °C for 2 min. Add 0.5 mL fibroblast medium to stop reaction and collect the cell suspension into a 15 mL tube. Centrifuge at 1,000 rpm for 5 min at room temperature. Discard supernatant and resuspend in 1 mL of human ESC-dFBS medium and count the cells. Seed 2–4 × 10^5 cells per 10 cm dish on a fibroblast feeder layer (*see* **Note 10**) and culture in human ESC-dFBS medium. Incubate the cells in 37 °C, 5 % CO_2 incubator. Centrifuge the remaining urine cells at 1,000 rpm for 5 min at room temperature. Aspirate the supernatant and suspend with 1 mL DPBS. Centrifuge again at 1,000 rpm for 5 min at room temperature and discard supernatant. Label it and store at −80 °C. This will be used for iPSC identification.

12. On Day 10, 2 days after the infected urine cells were split onto 10 cm dish, change fresh human ESC-dFBS medium, supplemented with 1 mM VPA and VC. Continue to incubate cells for 7 days and renew the medium daily.
13. On Day 16, some colonies would appear. However, they probably are "fake clones." Renew the medium daily with fresh mTeSR1 medium, supplemented with 1 mM Vc only. Stop adding VPA (*see* **Note 11**).
14. From Day 16–25, colonies will grow, so that the "fake clones" are easy to distinguish. At the same time, ESC-like colonies may appear.
15. From Day 25–30, those ESC-like colonies that are sufficiently large and identifiable could be picked mechanically (*see* **Note 12**). One or two days before picking colonies, prepare a 12-well plate with fibroblast feeder layers cultured in fibroblast medium. Aspirate the medium and wash with DMEM/F12 three times. Add 1 mL human ESC medium to each well and place the plates back in incubator. Burn the tip of a disposable Pasteur pipette until it becomes flexible. Stretch the tip and make it cuspidal with a tweezers. Burn the position away from the apex of 2–3 cm to make a small angle. Cut an intact colony into appropriate pieces under a microphone. Use a 200-μL pipettor to pick up all the pieces and deposit in one cell of 12-well plate. After finishing picking up all the colonies, incubate the cells under 5 % CO_2 at 37 °C, overnight and leave undisturbed for 2 days (*see* **Note 13**).

3.3 Characterization of iPS Cells

This part can be done according to a kit. Here we use a Sidansai Alkaline Phosphatase Staining Kit for this example.

3.3.1 Alkaline Phosphatase (AP) Staining

1. iPS cells are cultured on fibroblast feeder layers in a 24-well plate. When the cell clones become round enough (about 4–5 days after passaging), the AP staining can be started.

2. Aspirate the medium and wash with PBS 2–3 times.
3. Fix cells with 4 % paraformaldehyde (PFA) 1–2 min (*see* **Note 14**).
4. Aspirate PFA and wash with PBS twice.
5. Aspirate PBS and wash with TBST.
6. Prepare staining reagent, A liquid–B liquid–C liquid = 50 μL:50 μL:400 μL (provided in the kit).
7. Add abundant staining reagent and stand in dark at room temperature for 10–15 min.
8. Aspirate staining reagent and wash with PBS once. Keep cells in PBS and observe staining result under microscope.

3.3.2 Immunofluorescence Microscopy

1. Put a cover slip into each well of a 24-well plate, coat with250 μL 0.1 % Gelatin, and incubate at 37 °C, overnight. The next day, thaw a vial of fibroblast feeder cells (1.5×10^6 cells) onto glass coverslips and incubate under 5 % CO_2 at 37 °C. The immunofluorescence should not be started until the clones have attached well and are sufficiently large and with proper density.
2. Aspirate the medium and wash with PBS (*see* **Note 15**).
3. Fix iPS cells with 4 % paraformaldehyde for 30 min, 0.5 mL per well, and wash with PBS three times.
4. Block and permeabilize iPS cells in blocking solution for 2 h at room temperature. Prepare diluting primary antibodies in blocking solution and sealing films.
5. Stick the sealing film to the cover of the 24-well pate, and label primary antibodies. Add 20 μL of each primary antibody on the sealing film. Pry the glass coverslips from the 24-well plate with fine-tipped tweezers. Carefully attach the side with iPS cells to the antibody. Each antibody could take 2–3 repetitions. Incubate at room temperature for 1 h (*see* **Note 16**).
6. Pry glass coverslips onto the 24-well plate and wash three times with PBS in a shaker, 30 rpm, 5 min. Prepare diluting secondary antibodies in blocking solution. From this step onwards all operations need to be conducted away from light.
7. Add 250 μL secondary antibodies in each well of the 24-well plate. Incubate at room temperature for 2 h in dark place.
8. Wash with PBS three times in a shaker, 30 rpm, 5 min.
9. Cells were stained with DAPI for 5 min, 0.5 mL per well. Wash with PBS in a shaker, 30 rpm, 5 min.
10. Aspirated PBS and add fresh PBS. Pry glass coverslips onto a glass slide. Three to four coverslips per slide. Stored at 4 °C.
11. Observe and photograph under a confocal microscope.

3.3.3 RT-PCR for Marker Genes

1. Cell collection: Aspirate the medium and wash with DMEM/F12. Digest by 0.5 mL 0.25 % trypsin for 2 min at 37 °C. Observe cells under microscope, if the clones become round and loose, add 0.5 mL fibroblast medium to stop digestion. Blow off the colonies with a pipette. Then transfer the cell suspension into a 15 mL tube. Centrifuge the mixture at 1,000 rpm for 5 min at room temperature. Discard the supernatant and resuspend with 1 mL DPBS. Transfer the cell suspension into a 1.5 mL tube. Centrifuge the mixture at 1,000 rpm for 5 min at room temperature. Discard supernatant. Store at −80 °C or directly use to extract RNA.
2. Total RNA was extracted using TRIzol Plus RNA Purification Kit and treated with DNA-free kit to remove genomic DNA contamination.
3. 1 μg total RNA from each sample was used for reverse transcription with PrimeScript RT reagent Kit.
4. Quantitative PCR is then performed using a SYBR Green Premix EX Taq™ and Thermal Cycler Dice™ Real Time System to detect the expression of endogenous pluripotent genes and the silence of exogenous transgenes. Beta actin was used for normalization and all items were measured in triplicate.

3.3.4 Integration of the Exogenous Transgenes by Semiquantitative PCR

1. Genomic DNA was extracted using the Universal Genomic DNA Extraction Kit.
2. The exogenous transgene primers were used to amplify genomic DNA of the indicated iPS clones, primary cells, water, and ES cells were included as controls.
3. PCR products were conducted agarose gel electrophoresis and then visualized the DNA fragments on a long wave UV light box and photographed with a Polaroid camera.

3.3.5 Karyotype Analysis

1. iPS cells passaged after 4–5 days are selected to conduct chromosome karyotype analysis. Add medium containing 200 ng/mL colchicine and incubate at 37 °C for 3 h.
2. Aspirate the medium and add 0.5 mL 0.05 % trypsin, digesting for 2 min at 37 °C. Add 0.5 mL fibroblast medium to stop digestion, and transfer cell suspension into a 15 mL tube, centrifuge for 5 min, at 12,000 rpm.
3. Add 5 mL hypotonic solution (preheat to 37 °C before use) and incubate at 37 °C for 30 min.
4. Add 1 mL fixing solution dropwise into hypotonic solution. Fix at room temperature for 5 min. Centrifuge at 1,200 rpm for 5 min.
5. Aspirate the supernatant. Cells are fixed in 5 mL fixing solution for 10 min. Centrifuge at 1,200 rpm for 5 min. Aspirate the

supernatant, leaving some fixing solution. Resuspend cells and transfer cell suspension dropwise onto a wet slide (precooled at 4 °C before use).

6. After air drying, age for 3 days at 37 °C. Digest with 0.1 % trypsin at 37 °C for 5 min. Wash by tap water. Stain by 1 % Giemsa for 15 min.
7. After washing again with tap water and natural withering, metaphased cells with dispersed chromosomes are to be selected for chromosome counting and photography under the microscope.

3.3.6 Bisulfite Sequencing Analysis

1. Genomic DNA is extracted using the Universal Genomic DNA Extraction Kit.
2. 700 ng genomic DNA is digested in 1 μL EcoRV for 6 h at 37 °C.
3. Place the tubes with digested DNA in a boiling water bath for 10 min and then transfer on ice for 5 min.
4. Add 4 μL 2 M NaOH. Incubate at 50 °C for 15 min.
5. Prepare three 1.5 mL tubes for each sample. Add 300 μL mineral oil. Put them on ice for precooling.
6. Add 50 μL 2 % low gelling temperature agarose (LMP) to DNA mixture. Mix the sample by pipetting the sample up and down.
7. Add 11 μL DNA-LMP mixture to precool mineral oil. The mixture would form a globule and sink to the bottom.
8. Place the tubes with globule on ice for 15 min to solidify the globules.
9. Add 500 μL 2.5 M sodium metabisulfite solution (*see* **Note 17**).
10. Place the tubes at 50 °C for 12 h in a dark place.
11. Take out the tubes and discard the liquid with a waste liquor pump, only with the globule left.
12. Wash the globules with 1 mL 1× TE, pH 8.0 three times, with 10 min standing between each interval.
13. Wash with 500 μL 0.2 M NaOH twice, with 15 min standing between each interval.
14. Wash with 1× TE three times.
15. Discard the TE and add 100 μL fresh 1× TE into the tube. Stored at 4 °C.
16. The promoter regions of Oct4 and Nanog genes are amplified by nested-PCR with the DNA globule as template using Taq (*see* **Note 18**). The first round PCR products are used as the templates of the second round nested-PCR (*see* **Note 19**).

17. The resultant PCR products are cloned into pMD19-T Simple Vector.
18. Ten clones of each sample are sequenced.
19. Align sequencing results with the stand gene. If the cytosine of a CpG island remains unchanged, this site is methylated. If the cytosine becomes thymine, this site is unmethylated.

3.3.7 In Vitro Differentiation

iPS cells can form embryoid bodies (EB) when cultured in suspension in vitro. It is an access to the identification of in vitro differentiated potential. This part is based on ref. (9).

1. iPS cells are cultured on MEF feeder layers with human ESC medium until the density reaches 80 % confluency.
2. Aspirate the medium and wash with DMEM/F12. Add 500 μL Collagenase IV and digest in incubator for 50 min.
3. iPS clones are collected in a 15 mL tube.
4. Add DMEM/F12 to 5–6 mL, so that these clones could be precipitated. Repeat this step two times.
5. Resuspend with EB medium (*see* **Note 20**) and transfer to bacterial culture dish in the EB medium. Stand for 3 days. The medium is changed every other day.
6. After 8 days as a floating culture, embryonic bodies (EBs) are transferred to BD Matrigel-coated 6-well plate and cultured in the same medium for another 8 days. The medium is replaced every other day.
7. When cell density reaches 90 % confluency, cells are harvested for RNA extraction.
8. Quantitative PCR is performed using a SYBR Green Premix EX Taq™ and Thermal Cycler Dice™ Real Time System to detect the expression of endogenous pluripotent genes and marker genes of three germ layers.

3.3.8 Teratoma Formation

Teratoma formation is a way to test the in vivo differentiated potential of iPS cells. This part refers to ref. (9).

1. iPS cells on BD Matrigel with mTeSR1 medium are treated with 2× dispase for 2–3 min at 37 °C.
2. Collect cells by scraping with 1 mL pipette tips and transfer into a 15 mL tube. Centrifuge at 1,000 rpm for 5 min.
3. After centrifugation, cell pellets are resuspended in 300 μL diluted Matrigel (Matrigel:DMEM/F12 = 1:2). Transfer into 1.5 mL tubes. Keep on ice (*see* **Note 21**).
4. 200 μL cell suspensions are injected subcutaneously and 100 μL intramuscularly into the right hind leg of immune-compromised NOD-SCID mice.

5. Tumors can be excised 8–10 weeks later.
6. Tumors is then fixed and embeded in paraffin and then sectioned and stained with hematoxylin/eosin.

3.3.9 Identification of Mutant Gene

If the disease is caused by gene mutation, the identification of defect gene is necessary.

1. Genomic DNA was extracted using the Universal Genomic DNA Extraction Kit.
2. DNA sample are to be verified by sequencing.

4 Notes

1. Penicillin/Streptomycin helps to protect from bacterial contamination during primary urine cells isolation. However, if patients are susceptible to aminoglycoside antibiotics, it is better to choose penicillin rather than penicillin/streptomycin.
2. From step 4, the following steps should be handled in cell culture room. It is recommended that the cell isolation should be taken immediately after sample collection. If the experiment has to be postponed unavoidably, urine samples can be kept at 4 °C or on ice for some time.
3. Urine samples usually include many impurities such as squamous cells and blood cells. Generally, female urine samples contain more sediment than those of male samples. These cells will disappear gradually during the process of medium change.
4. P1 urine cells can be split directly onto a 6-well plate and used for infection. The best efficiency of iPS cell generation is obtained when primary urine cells at P1–P4.
5. Every transcription factor needs a dish of HEK 293T cells to package. There are totally four factors. Additionally, pMXs-GFP as a control for checking transfection efficiency needs another dish of HEK 293T cells. Therefore, five dishes are needed.
6. The pipetting after adding 2× HBS needs to be done violently and the following 2 min standing is very important, so that there will form DNA-calcium phosphate sediment. These sediments will enter 293T cells through endocytosis.
7. If the urine cell collection is synchronized with virus package, then directly split urine cells and seed appropriate cells in a 6-well plate on Day 3. If the urine cells have been frozen in liquid nitrogen, then on Day 1, urine cells must be thawed in a 6 cm dish. On Day 3, split the urine cells and seed appropriate cell number in a 6-well plate.

8. The transfection efficiency is extremely important for later infection of urine cells. If the number of transfected HEK 293T cells is very small or the fluorescence signal is weak, check for a problems or issues with the reagent calcium phosphate transfection, HEK 293T cell state, the operation process, and so forth.
9. Since HEK 293T cells do not stick to the plate bottom very closely, medium should be dropped onto the sides of the dishes, so that the cells will not detach.
10. Mouse embryonic fibroblasts (MEFs) are a kind of supporting layers to keep hESC/hiPSC expansion as well as prevent spontaneous differentiation. The feeders are cultured in fibroblast medium and should only be plated 1–2 days before splitting the infected urine cells. Before use, aspirate the fibroblast medium and wash three times with DMEM/F12. Since the fibroblast medium contains FBS which would induce spontaneous differentiation of ESC/iPSC.
11. If using mTeSR1 instead of human ESC-dFBS, the medium can be started a day earlier. It all depends on the condition of feeder layers. If the MEF feeder layer has become thin and many cells die and detach, then replace the medium immediately. Generally, the feeder layer can last for 7 days. VPA could only be added for 7 days, since it is toxic to the reprogramming cells and MEF feeder cells.
12. When ESC-like colonies first appear, they are relatively small. Do not pick them until they grow for about 1 week and become sufficiently large. Conversely, do not let them overgrow; otherwise, they will probably differentiate.
13. The whole process of picking up colonies is outside of the biological safety cabinet, under the microphone with the cells exposed to the air in cell culture room. Before picking colonies, the cell culture room should be sterilized using ultraviolet lighting for 30 min. During the picking period, avoid other people walking into the room.
14. Excessive fixation would deactivate the Alkaline Phosphatase. Therefore, cell should not be fixed for more than 2 min.
15. Do not blow away clones when adding PBS by Pasteur pipette. It is better to add PBS against walls rather than directly to the cells.
16. In order to prevent the antibodies from becoming dry, the cover with glass coverslips on the sealing films should be put in a wet box.
17. Make sure that the globule is blown to suspend on the interface between mineral oil and sodium metabisulfite solution.

18. The first round nested-PCR adopted the following conditions: initial denaturation for 5 min at 95 °C; 35 cycles of 94 °C for 30 s, 57 °C for 45 s, 72 °C for 50 s; and followed by 72 °C for 7 min.
19. The second round nested-PCR adopted the following conditions: initial denaturation for 5 min at 95 °C; 35 cycles of 94 °C for 30 s, 58 °C for 45 s, 72 °C for 50 s; and followed by 72 °C for 7 min.
20. Use a Pasteur pipette to resuspend, so that the cells could retain intact clones.
21. Matrigel would soon curdle at room temperature.

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Patient-Specific Induced Pluripotent Stem Cell Models: Characterization of iPS Cell-Derived Cardiomyocytes

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Abstract

Despite significant advances in medical treatment, cardiovascular disease is still a major cause of morbidity and mortality in advanced countries. To improve the outcome, the further promotion of basic cardiovascular science has a pivotal role for the developing novel therapeutic approach. However, due to the inaccessibility of human heart tissue, we couldn't obtain the sufficient amount of patient's heart tissues. The discovery of human-induced pluripotent stem cells (iPSCs) is highly expected to provide the breakthrough to this obstruction. Through the patient-specific iPSCs-derived cardiomyocytes, we could analyze the patient-specific heart diseases directly and repetitively. Herein we introduce the outline of creation for cardiac disease modeling using patient-specific iPSCs. Within several topics, we present the actual representative methodologies throughout the process from the derivation of cardiomyocytes to those of functional analysis.

Keywords: iPS cell, Cardiomyocyte, Purification, Electrophysiology

1 Introduction

In the mid of 2000s, the generation of induced pluripotent stem cells (iPSCs) brought a great impact on stem cell biology (1, 2). The major expected role of iPSCs could bear mainly consists of two parts, one is as a robust resource for the regenerative cell therapy and the other is as a tool for the creation of human genetic disease modeling. There are already many reports showing that iPSCs were generated from a variety of patients and those iPSC-derived differentiated cells successfully reproduced the disease phenotypes, and this type of analysis has shed light on the further understanding of the underlying disease mechanism and subsequently provided the cue for developing novel therapeutic approach (3). Along with the accumulation of individual research outcomes, the comprehensive research projects such as high-throughput drug screening by using patient-specific iPSC-derived cells have been designed, and actually some intriguing results are appealing (4–6). We have previously reported the disease modeling for long QT syndrome, a hereditary

arrhythmic disease with high incidence, by using patient-specific iPSCs (7). We clarified the molecular mechanism of the disease pathogenesis in the patient with novel KCNQ1 mutation and verified the appropriate medication through the drug response examination on electrical activity of the patient-specific iPSC-derived cardiomyocytes. Herein we introduce the outline how to examine the patient-specific iPSC-derived cardiomyocytes, which covers the whole process from the differentiation into cardiomyocytes from iPSCs to functional analyses.

2 Materials

2.1 Equipment

100 mm tissue culture dish (Falcon® 353003).

6-well tissue culture plate (Falcon® 353046).

24-well tissue culture plate (Falcon® 353047).

96-well tissue culture plate (Falcon® 353072).

Ultra-low attachment culture dishes, 100 mm petri dish (Corning® 3262).

15 ml conical tube (Falcon® 352096).

50 ml conical tube (Falcon® 352070).

1 ml plastic disposable pipette (Falcon® 357521).

2 ml plastic disposable pipette (Falcon® 357507).

5 ml plastic disposable pipette (Falcon® 357543).

10 ml plastic disposable pipette (Falcon® 357551).

25 ml plastic disposable pipette (Falcon® 357525).

50 ml plastic disposable pipette (Falcon® 357550).

20 μl filter tip (NIPPON Genetics® FGF-20S2).

200 μl filter tip (NIPPON Genetics® FGF-7032).

1,000 μl filter tip (NIPPON Genetics® FGF-10002).

Pasteur pipette (IWAKI® IK-PAS-9P).

0.22 μm pore size filter (Millipore®, SCGPT10RE).

40 μm cell strainer (Falcon® 352340).

100 μm cell strainer (Falcon® 352360).

40 μm cell strainer (Falcon® 352340).

Ultra-low attachment culture dishes, 100 mm petri dish (Corning® 3262).

Magnetic bar (IKAFLON® 1572000).

Magnetic stirrer (AS ONE® CS-1).

4-well chamber slide (Nunc® Lab-Tek®II 154534).

Micro cover glass (Matsunami Glass® C024501).

LSM510 Meta Confocal Microscope (ZEISS).

Multi-electrode array (MEA) chips.

Axopatch 200B (Molecular Devices).

Digidata 1440A (Molecular Devices).

pClamp software 10.2 (Molecular Devices).

Glass pipette (Harvard Apparatus).

Matsunami glass bottom dish (Matsunami).

Magnetic stirrer bar (AS ONE® CS-1).

35 mm glass-bottomed dish (IWAKI).

2.2 Materials

Collagenase, type IV, powder (GIBCO® 17104-019).

DMEM/F12 (Sigma-Aldrich® D6421).

1 mg/ml of collagenase, type IV solution: Dissolve 100 mg of collagenase IV in 100 ml of DMEM/F12 and through with a 0.22 μm pore filter. Aliquot and store at 4 °C up to a week.

mTESR1 (STEMCELL TECHNOLOGIES® 05850).

Sterile-filtered water (Sigma-Aldrich® W3500).

Recombinant human BMP 4 (R&D Systems® 314-BP-050).

Blebbistatin (Sigma-Aldrich® B0560).

Stempro34 (GIBCO® 10639-011).

Ascorbic acid (Sigma-Aldrich® A5960).

GlutaMAX (GIBCO® 35050-061).

Recombinant human Activin A (R&D Systems® 328-AC-050).

IWR-1 (Sigma-Aldrich® I0161).

DMEM, no glucose (GIBCO® 11966-025).

L-Lactic acid (Wako® 129-02666).

Lactic medium: 500 ml DMEM without glucose supplemented with 170 μl of L-lactic acid (final concentrate for 4 mM lactic acid solution).

Phosphate-buffered saline without calcium and magnesium (PBS) (WAKO® 045-29795).

2.5 % trypsin (GIBCO® 15090).

1 % collagenase type IV solution: dissolve 10 mg of collagenase IV in 10 ml of PBS, and through with a 0.22 μm pore filter. Aliquot and store at 4 °C up to a week.

Ads buffer: 116 mM NaCl, 20 mM HEPES, 12.5 mM NaH_2PO_4, 5.6 mM glucose, 5.4 mM KCl, and 0.8 mM $MgSO_4$; pH 7.35.

Dissociation buffer: Add 100 μl of 1 % collagenase type IV solution to 100 μl of 2.5 % Trypsin, then fill up to 1 ml by Ads buffer.

4 % paraformaldehyde (PFA) (MUTO PURE CHEMICALS® 33111).

Triton-X (Sigma-Aldrich® T9284).

0.1 % Triton-X: 10 μl of Triton-X in 10 ml of PBS.

Trizma® base (Sigma-Aldrich® T6066).

Tween® 20 (Sigma-Aldrich® P1379).

10× TBS: Dissolve 24.2 g of Trizma® base and 80 g of NaCl in 1,000 ml deionized water, pH 7.6.

1× TBST (1× TBS/0.1 % Tween® 20): Add 100 ml of 10× TBS to 1 ml of TWEEN® 20, then fill up to 1.,000 ml by deionized water.

ImmunoBlock (DS Pharma Biomedical® CTKN001).

Fibronectin (1 mg/ml) (Sigma-Aldrich® F1141).

Fibronectin coating solution (50 μg/ml): Dilute 1 mg/ml of fibronectin by PBS.

DAPI (Molecular Probes® D21490): To make a 5 mg/mL DAPI stock solution: Dissolve the contents of 10 mg in 2 mL of deionized water. For long-term storage, the stock solution can be aliquoted and stored at ≤ -20 °C. For short-term storage, the solution can be kept at 2–6 °C, protected from light. Dilute the DAPI stock solution to 300 nM in PBS.

E4031 (M5060; Sigma-Aldrich).

Chromanol 293B (C2615; Sigma-Aldrich).

Barium chloride (Fluka 34252; Sigma-Aldrich).

Isoproterenol hydrochloride (I6504; Sigma-Aldrich).

Propranolol hydrochloride (P0884; Sigma-Aldrich).

Amphotericin B (Nacalai Tesque 02743-04).

Fluo-4AM (Invitrogen).

Caffeine (SIGMA-ALDRICH).

2.3 Primary Antibodies

Anti-a-Actinin (Sigma-Aldrich® A7811).

Anti-ANP (Santa Cruz® sc-20158).

Anti-MHC (Developmental Studies Hybridoma Bank® MF20).

Anti-TNNT (Thermo Scientific®, NeoMarkers®, 13-11).

Anti-GATA4 (Santa Cruz® sc-1237).

Anti-NKX2.5 (Santa Cruz® sc-8697).

2.4 Secondary Antibodies

Alexa Fluor 488 goat anti-mouse IgG.
Alexa Fluor 594 goat anti-rabbit IgG (all Life Technologies®).

3 Methods

3.1 Cardiac Differentiation of Human iPSCs

With respect to the disease modeling using iPSC-derived cardiomyocytes, to identify the cardiac differentiation protocol with highly reproducibility in many independent iPSC lines is a crucial issue. Thus far, several methods for cardiomyogenesis from iPSCs have been reported, though some were not universal in many independent iPSC lines (8). According to the summary of previous reports, the fact that BMP/Activin and Wnt signaling have a pivotal role for myocardial differentiation is widely accepted and most of current methods are the modification of these signaling manipulation. Herein we introduce one of the representative methods for cardiac differentiation from iPSCs using the stimulation of BMP/Activin signaling in early phase and the inhibition of Wnt signaling in later phase (9).

1. hiPSCs are split at 1:10 ratios, using collagenase type IV (1 mg/dl) as above and grown for 7 days, at which time they reached ~85 % confluence.
2. More than 100 μm-sized splitting iPSC clusters are gathered by 100 μm cell strainer and transfer to 100 mm ultralow attachment culture dish with 10 ml of mTESR1 supplemented with 10 ng/ml of BMP4 and 5 μM of Blebbistatin (day 0).
3. After 24 h incubation, embryoid body (EB) formation should be confirmed and change the medium to Stempro 34 supplemented with 50 μg/ml of ascorbic acid, 2 mM of GlutaMAX, 10 ng/ml of BMP4, and 25 ng/ml of Activin A and incubate the dish for 48 h (day 1–2).
4. Change the fresh Stempro 34 supplemented with 50 μg/ml of ascorbic acid and 2 mM of GlutaMAX. Remove BMP4 and Activin A at this time. Incubate the dish for 36 h (day 4–4.5).
5. Change the fresh Stempro 34 supplemented with 50 μg/ml of ascorbic acid and 2 mM of GlutaMAX and add 2.5 μM of IWR-1; then incubate the dish for 96 h (day 4.5–8.5).
6. Change the fresh Stempro 34 supplemented with 50 μg/ml of ascorbic acid and 2 mM of GlutaMAX. Remove IWR-1 at this time (day 8.5 ~).
7. Change the fresh medium every other day and beating EBs should become visible 2–3 weeks after the induction of differentiated culture condition (*see* **Note 1**).

3.2 In Vitro Purification of iPSC-Derived Cardiomyocytes

iPSC-derived differentiation cells contain wide variety of cells including endodermal cells, mesodermal cells including cardiomyocytes, and ectodermal cells. To investigate accurately the characteristics of iPSC-derived cardiomyocytes, it should be adequate to obtain highly purified cardiomyocytes. Herein we introduce the sophisticated method for in vitro purification of iPSC-derived cardiomyocytes utilizing the cardiomyocyte-specific metabolism (10). In spite of very simple procedure, we can obtain extremely high purified cardiomyocytes.

1. Prepare 18–25 days post-induction in EBs which contain more than 20 % in the incidence of spontaneous beating.
2. Transfer to 50 ml conical tube through 40 μm cell strainer, and wash EBs stayed on cell strainer twice with PBS.
3. Wash 100 mm culture dish twice with PBS and completely remove residual culture medium.
4. Retransfer EBs to 100 mm culture dish in 10 ml lactic medium.
5. 2 days post first change of lactic medium, break up the cells by pipetting up and down several times, and then transfer to 50 ml conical tube through 40 μm cell strainer to remove the medium containing dead cells.
6. Retransfer EBs to 100 mm culture dish in fresh 10 ml lactic medium.
7. 3 days post second change of lactic medium, exchange to fresh 10 ml lactic medium by the same procedure 5 and 6.
8. 5 days post third change of lactic medium, add 5 ml of cardiac differentiated medium.
9. 2 days post addition of cardiac differentiated medium, break up the cells by pipetting up and down several times, then transfer to 50 ml conical tube through 40 μm cell strainer to remove the medium containing dead cells.
10. Retransfer EBs (purified myocardial clusters) to 100 mm culture dish in fresh cardiac differentiated medium.

3.3 Single-Cell Enzymatic Dissociation of iPSC-Derived Cardiomyocytes

1. Collect the cell suspension into a 15 ml conical tube.
2. Centrifuge at 160 × *g* for 5 min, and then discard supernatant.
3. Resuspend a pellet of purified myocardial clusters with 1 ml of dissociation buffer, and transfer to a 1.5 ml tube.
4. Dissociate using magnetic stirring for 30 min in a 37 °C, 5 % CO_2 incubator.
5. Centrifuge the cells, and then discard supernatant.
6. Add 1 ml of differentiated medium, and break up the cells by pipetting up and down several times.
7. Transfer to an adequate-sized dish by each assays.

3.4 Immunofluorescence Staining

1. Dissociated hiPSC-derived cardiomyocytes were plated onto fibronectin-coated Lab-Tek® II 4-chamber glass slides and were allowed to grow for 3 days in differentiated medium.
2. Cells were fixed with 4 % PFA for 30 min at room temperature, permeabilized with 0.1 % Triton-X for 5 min at room temperature, blocked in ImmunoBlock for 30 min at room temperature, and stained using the following primary antibodies and reagents which are 1:200 anti-a-Actinin, 1:100 anti-ANP, 1:100 anti-MHC, 1:100 anti-TNNT, 1:100 anti-GATA4, 1:100 anti-NKX2.5 overnight at 4 °C in ImmunoBlock.
3. Cells were washed four times, for 5 min, with 1× TBST, and then incubated for 1 h at room temperature in the dark with secondary antibodies 1:1,000 Alexa Fluor 488 goat anti-mouse IgG or 1:1,000 Alexa Fluor 594 goat anti-rabbit IgG (Life Technologies) in ImmunoBlock.
4. Cells were washed again as above, nuclei were stained with DAPI, and micro cover glasses were attached to Lab-Tek® II 4-chamber glass slides without the outer frame and imaged with an LSM510 Meta Confocal Microscope.

3.5 The Identification for Electrophysiological Activity of Patient-Specific iPSC-Derived Cells

One of the great benefits for the utilization of patient-specific iPSC-derived cells is to examine directly and repetitively the cellular function of patient-specific alive cells. Here we introduce four methods for the evaluation for electrophysiological activity of iPSC-derived cardiomyocytes (*see* **Notes 2** and **3**).

3.5.1 Field Potential Recordings Using the On-Chip Multi-electrode Array System

The on-chip multi-electrode array system is convenient for analyzing the electrophysiological activity of iPSC-derived cardiomyocytes. We characterize the electrical properties of iPSC-derived cardiomyocytes through the record of field potential which is regarded as similar to body surface electrocardiogram in clinical practice. Since the record can be obtained from cell clusters, it would reflect the electrical status nearby myocardial tissue level, whereas the patch clamp is recorded by a single cell. Furthermore, we can simply evaluate the drug response of multiple myocardial clusters at once, and this method could be a platform for high-throughput drug screening.

1. Multi-electrode array (MEA) chips from Multi Channel Systems (Germany) are coated with 0.001 % fibronectin solution and incubated at 37 °C overnight.
2. Remove the residual fibronectin solution and purified myocardial clusters are plated with culture medium and incubated at 37 °C for several days.
3. Transfer the medium to serum-free once a day before recording.

4. MEA measurements are performed at 37 °C. Obtain the data for approximately 5 min per experiment and subsequently analyze it with MC_Rack (Multi Channel Systems).
5. Record multiple parameters such as beating frequency, the amplitude of each wave, and field potential duration (FPD) defined as the time interval between the initial deflection of the field potential and the maximum local T wave. FPD measurements are usually normalized (corrected FPD: cFPD) to the activation rate using Bazett's correction formulae: $cFPD = FPD/(RR\ interval)^{1/2}$, where RR indicates the time interval (in seconds) between two consecutive beats (7).
6. Several drugs such as E4031, chromanol 293B, barium chloride, isoproterenol hydrochloride, and propranolol hydrochloride are prepared as 1 or 10 mM stock solutions.
7. After the baseline of field potentials are recorded for 5 min, add the drug to the medium. After 5–10 min of incubation, measure the field potentials for 5–10 min.
8. Perform MEA recordings by investigators blinded to the genotype of the cells.

3.5.2 Patch Clamp Analysis

Whole-cell patch clamp recordings of iPSC-derived cardiomyocytes can be measured using almost the same methods and materials for cardiomyocytes isolated from animals. However, the size and the channel currents of cardiomyocytes derived from iPS cells are usually smaller than those of animal cardiomyocytes. Therefore, it may be difficult to measure currents of some types of channel.

3.5.3 IK+ Measurement

1. Prepare glass bottom dish coated with coating solution (0.1 % gelatin and 2 % fibronectin diluted by PBS). Coating needs at least 15 min.
2. Dissociate iPSC-derived cardiomyocytes to single cells (*see* Section 3.3). Place dissociated cardiomyocytes onto coated glass bottom dish and incubate 37 °C for 60–120 min.
3. Remove the culture medium. Fill normal Tyrode's solution (NaCl 135 mM, KCl 5.4 mM, NaH_2PO_4 0.33 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 0.5 mM, HEPES 5.0 mM, and glucose 5.5 mM, pH 7.4 adjusted with NaOH) into the iPSC-derived cardiomyocytes attached dish. Set the dish on the stage of microscope of patch clamp system. Axopatch 200B, Digidata 1440A, and pClamp 10.2 (Molecular Devices, USA) can be used for data amplification, acquisition, and analysis, respectively.
4. The resistance of glass pipette is 3–5 MΩ (M ohm) after filling with the internal pipette solution (KOH 60 mM, KCl 80 mM, aspartate 40 mM, HEPES 5 mM, EGTA 10 mM, Mg ATP

5 mM, sodium creatinine phosphate 5 mM, and $CaCl_2$ 0.65 mm, pH 7.2 adjusted with KOH). After sealing and rupture of iPSC-derived cardiomyocytes, perfuse external solution for IK+ recording (*N*-methyl-D-glucamine 149 mM, $MgCl_2$ 5 mM, HEPES 5 mM, nisoldipine 0.003 mM, pH 7.4).

5. Record the currents using voltage clamp protocol. Peak current is recorded with 3 s depolarizing steps from a holding potential of −60 mV to potentials ranging from −60 to +60 mV in 10 mV increments. Tail current is recorded with a 2 s repolarization phase to −40 mV following the step pulse. Pulse frequency was 0.1 Hz.
6. For slow rectifier K+ current (IKs) recording, subtract the current recorded after applying chromanol 293B (10 mM) from IK+. Subtract the current recorded after administrating E4031 (10 mM) from IK+ for rapid rectifier K+ current (IKr) recording.
7. Internal and external solutions for INa or ICa-L recording are given below.

Internal pipette solution for INa+ (mM); CsCl2 130, MgCl2 2, ATP-2Na 5, HEPES 10, EGTA 10, TEA-Cl 20 (pH 7.3 adjusted with CsOH).

External solution for INa+ recording (mM); NaCl 135, CsCl 5.4, $MgCl_2$ 0.53, $CaCl_2$ 2, HEPES-NaOH 5, glucose 5.5, $CdCl_2$ 0.1 (pH 7.4 adjusted with NaOH).

Internal pipette solution for ICa2+ -L (mM); CsOH 60, CsCl 80, aspartate 40, HEPES 5, Mg-ATP 5, phosphocreatine 5, EGTA 10, $CaCl_2$ 0.65 (pH 7.4 adjusted with CsOH).

External solution for ICa^{2+}-L recording (mM); choline chloride 140, CsCl 5, $MgCl_2$ 1, $CaCl_2$ 1.8, glucose 10, HEPES 10 (pH 7.4 adjusted with CsOH).

3.5.4 Ca^{2+} Imaging Assay

Ca^{2+} imaging for iPSC-derived cardiomyocytes has been performed by the same manner as the conventional measurement for other animal myocardial cells. Since the cellular size of human iPSC-derived cardiomyocytes is significantly smaller than the myocardium of other species, the laser exposing time should be reduced by the minimum requirement and the laser intensity should be also adjusted to the proper grade.

1. Dissociated iPSC-derived cardiomyocytes are plated onto 1 % fibronectin-coated 35 mm glass-bottomed dishes (IWAKI) and grow for 2 days with the differentiated medium.
2. Incubate with 5 μM Fluo-4AM (Invitrogen) in Tyrode's solution at 37 °C and wait for loading into cytosol for 30 min.

3. Set up a confocal microscope (LSM 510 Duo, Carl Zeiss) with a $\times 40$ lens (NA = 0.75) for Ca^{2+} imaging.
4. Line scan analysis is acquired at a sampling rate of 2 ms per each line (obtain 10,000 times recordings in 20 s).
5. For the estimation of Ca^{2+} storage in sarcoplasmic reticulum, measure the amplitude of Ca^{2+} transients exposed by the rapid administration of 10 mM caffeine.

4 Notes

1. Distinct iPSC lines show different cardiac differentiation efficiency. It is difficult to compare the cardiac differentiation efficiency among different iPSC lines. Therefore, it is required to generate isogenic iPSCs to show if disease-specific iPSCs would show the difference of cardiac differentiation efficiency.
2. The maturity of iPSC-derived cardiomyocytes affects the result of electrophysiological examination and molecular analysis. To adapt the maturity among the subjects, culture condition should be unified such as differentiation method and differentiation period.
3. Temperature is an important factor to conduct electrophysiological examination. To obtain the data accurately, every equipment and solution should be kept at adequate temperature.

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Generation of Integration-Free Patient Specific iPS Cells Using Episomal Plasmids Under Feeder Free Conditions

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Abstract

Reprogramming somatic cells into a pluripotent state involves the overexpression of transcription factors leading to a series of changes that end in the formation of induced pluripotent stem cells (iPSCs). These iPSCs have a wide range of potential uses from drug testing and in vitro disease modelling to personalized cell therapies for patients. While viral methods for reprogramming factor delivery have been traditionally preferred due to their high efficiency, it is now possible to generate iPSCs using nonviral methods at similar efficiencies. We developed a robust reprogramming strategy that combines episomal plasmids and the use of commercially available animal free reagents that can be easily adapted for the GMP manufacture of clinical grade cells.

Keywords: Induced pluripotent stem cells, Episomal plasmids, Reprogramming

1 Introduction

Embryonic stem cells (ESCs) are isolated from the inner cell mass (ICM) of the blastocyst (1). These in vitro derivatives preserve the pluripotency of the ICM and can self-renew indefinitely under defined culture conditions. By harnessing the differentiating potential of pluripotent cells in vitro, the field of regenerative medicine observed the beginning of a new era. Many protocols have been developed showing that ESCs can be differentiated into cells of various lineages including neurons, cardiomyocytes, blood progenitors, hepatocytes, and retinal precursors (2–4). These cells are functional and can rescue disease phenotypes in animal models, reinforcing their potential for cellular therapy (4) and for in vitro drug testing studies (5). Despite all the potential of ESCs, there are challenges that need to be overcome. Primarily, there is the ethical issue of destroying human embryos in order to derive ESCs. Furthermore, cells differentiated from ESCs are not patient specific and if used in cell therapies, could pose a problem of immune rejection (4).

In 2006, Kazutoshi Takahashi and Shinya Yamanaka achieved a breakthrough with the generation of the first pluripotent stem cells (iPSCs) (6). These iPSCs were induced from mouse embryonic fibroblasts by the expression of four exogenous transcription factors: Oct3/4, Sox2, Klf4, and C-Myc (OSKM), known as the Yamanaka factors. A year later, the same group generated the first human iPSCs followed by two other groups (7–9). These iPSCs were found to have similar characteristics as ESCs including morphology, growth properties, expression patterns, and the ability to form teratomas containing tissue derivatives of the three germ lineages. Great interest now surrounds iPSCs as they avoid the destruction of human embryos and can be derived directly from patients' cells, thus bringing the possibility of autologous cellular therapies a step closer. iPSCs have also proved to be a valuable tool in basic research, allowing molecular mechanisms behind the diseased phenotype to be elucidated in vitro, which has been particularly vital where animal models do not faithfully recapitulate human disease (10). Such disease specific iPSCs have also found utility in drug testing (11). Despite the benefits, there are still doubts regarding the potential use of these cells in regenerative medicine. The major drawback is based on an important feature of these cells, their pluripotency. The dangers being that the iPSCs could gain a cancerous phenotype or residual undifferentiated cells could be introduced into patients potentially resulting in teratoma formation. Thus, from reprogramming and differentiation to purification of the differentiated cells, careful optimization of the entire process is essential before iPSCs can be used for patient therapies.

The original Yamanaka iPSC method entails the use of integrating retroviral vectors, which raises the possibility of insertional oncogenesis and is therefore not suitable for patient therapies. In the race for developing clinically translatable reprogramming methodologies, many started focusing on nonviral, integration free methods for generating iPSCs. The use of EBNA-1 based episomal plasmids has gained widespread appeal as it does not require the technical complexity of synthesizing mRNA, proteins, or Sendai virus. While early attempts using episomal plasmids were highly inefficient, significant improvements to Thomson's original protocol have since been made (12). In particular, Okita et al. have shown that the addition of a short hairpin against p53 and the replacement of C-Myc with L-Myc to the reprogramming cocktail of Oct3/4, Sox2, Klf4, and Lin28 resulted in over tenfold increase in efficiencies (13). Using these same episomal plasmids, we developed a highly reproducible method for reprogramming patient dermal fibroblasts at efficiencies comparable to the original retrovirus protocols (approximately 0.1 %) (6). Moreover, we eliminated the use of mouse embryonic fibroblasts (MEFs) which increases the risk of contamination with endogenous and adventitious agents and found that our protocol yielded similar efficiencies with or

without their use. Combined with the use of commercially available animal free reagents (14), the method described in this chapter can be easily adapted to generate good manufacturing grade (GMP) compliant iPSCs.

2 Materials and Equipment

2.1 Equipment

1. Centrifuge.
2. Inverted fluorescence microscope.
3. Microscope.
4. Stereomicroscope.
5. Water bath.
6. Haemocytometer or other cell counter.
7. Flow cytometer, such as CyAN.
8. NanoDrop ND1000 Spectrophotometer (NanoDrop™, ThermoScientific).
9. PCR cycler such as Bio-Rad.
10. Controlled rate freezing apparatus such as Mr Frosty (Nalgene).
11. Nucleofector 2b device.

2.2 Reprogramming Human Dermal Fibroblasts

1. Shinya Yamanaka episomal plasmids: pCXLE-hOct3/4-shp53-F (Addgene number 27077), pCXLE-hSK (Addgene number 27078), pCXLE-hUL (Addgene number 27080), and pCXLE-EGFP (Addgene number 27082). Plasmids should be diluted to 1 μg/ml using TE buffer or water and stored at −20 °C in small aliquots to avoid repeated freeze–thaws. Keep plasmids on ice during use.
2. Dulbecco's phosphate buffered saline without calcium and magnesium (Life Technologies).
3. TrypLE express (Life Technologies).
4. Fine tip plastic Pasteur pipette 3.5 ml draw individually wrapped, sterile (Appleton Woods).
5. Amaxa Nucleofector kit R (Lonza).
6. 0.1 % gelatin solution. Warm 25 ml of 2 % tissue culture grade gelatin solution type B (Sigma-Aldrich) at 37 °C water bath until solution is liquefied. Mix with 475 ml of PBS without calcium and magnesium and sterilize the solution with a 0.2 μm filter into a 500 ml sterile bottle. 0.1 % solution may be stored at 4 °C until use.
7. 1 M sodium butyrate solution. To a 1 g bottle of sodium butyrate (Sigma-Aldrich), add 9 ml of sterile tissue culture

grade water. Sterilize the solution with a 0.2 μm filter and aliquot into Eppendorf tubes. Store aliquots at −20 °C protected from light until use. Sodium butyrate is used at a working concentration of 0.5 mM, add 5 μl of 1 M sodium butyrate solution to 10 ml of medium.

8. Fibroblast medium: To high glucose DMEM with L-glutamine and sodium pyruvate (PAA cell laboratories), add 10 % fetal bovine serum (Life Technologies), 1 mM nonessential amino acids (NEAA) and 1× penicillin–streptomycin (10,000 IU PAA cell laboratories).
9. Geltrex LDEV-free hESC-qualified, reduced factor basement membrane matrix (Life Technologies). Store Geltrex stocks at −80 °C. For manageable aliquots, thaw 5 ml bottle of Geltrex overnight on ice in a 4 °C fridge. Pre-label 40× 15 ml centrifuge tubes, uncap and keep tubes on ice while aliquoting. Aliquot 122 μl of undiluted Geltrex to each tube. Re-cap and immediately store upright in a −20 or −80 °C freezer until use. To use, add 12 ml of cold DMEM/F12 directly to the Geltrex aliquot and pipette up and down to dissolve the Geltrex. Immediately transfer 1 ml per well of a 6-well plate for coating. Unused plates are stored wrapped in Parafilm at 4 °C for up to 1 week. Discard any plates that are dried out. Alternatively for animal free generation of iPSC, use vitronectin recombinant human protein (Life Technologies), Vitronectin XF (Stemcell Technologies), or Synthemax-R (Corning) as per manufacturer's instructions.
10. Complete mTESR1 medium (Stemcell Technologies). For 50 ml, add 10 ml of mTESR1 5× supplement and 0.5 ml of penicillin–streptomycin (PAA laboratories) to 40 ml of mTESR1 basal medium. Alternatively for animal free generation of iPSCs, use Essential 8 (E8) medium (E8 from Life Technologies or TeSR-E8 from Stemcell Technologies). Prepare medium as per manufacturer's instructions.

2.3 Growth, Maintenance, and Cryopreservation of Human iPSCs

1. Gentle cell dissociation reagent (Stemcell Technologies).
2. 500 μM Y27623 ROCK inhibitor (Calbiochem). To 1 mg bottle of Y27623 powder, add 5.91 ml of PBS or sterile tissue culture grade water. Sterilize using a 0.2 μm filter. Store aliquots protected from light at −20 °C until use. ROCK inhibitor is used at a working concentration of 10 μM. Add 20 μl of 500 μM ROCK solution to 1 ml of medium.
3. Bambanker serum free cell freezing medium (Lymphotec). Store at 4 °C and use undiluted.
4. Cell lifter with 19 mm blade and 180 mm handle individually wrapped (Corning).
5. DMEM/F12 (Life Technologies).

2.4 Characterization of Human Pluripotent Stem Cells

1. 4 % paraformaldehyde (Sigma-Alrich).
2. Goat serum (Life Technologies).
3. Bovine serum albumin (Sigma-Aldrich).
4. 100 % methanol.
5. Blocking solution: PBS with 5 % goat serum and 0.1 % bovine serum albumin.
6. Primary antibodies Oct3/4, TRA-1-60, TRA-1-81, SSEA-1, and SSEA-4 (Santa Cruz Biotechnology).
7. Secondary antibody: Alexa Fluor 488 goat anti-mouse IgG, IgM (Life Technologies).
8. DAPI (Sigma-Aldrich).
9. Alkaline phosphatase staining kit (Millipore).
10. TRIzol (Life Technologies).
11. Chloroform.
12. DMEM/F12 (Life Technologies).
13. Superscript III cDNA conversion kit (Life Technologies).
14. SYBR green.
15. Accutase enzyme cell detachment medium.
16. FACS buffer: 2 % FCS in PBS.

3 Methods

All of the following procedures, unless otherwise specified, are performed in biosafety hoods using standard laboratory practices to minimize exposure to animal pathogens and carried out at room temperature unless otherwise specified. All cells were cultured in tissue culture coated flasks or plates (Corning) at 37 °C in humidified incubators in 5 % CO_2/95 % air. All patient fibroblasts were either purchased from ATCC or Coriell cell repositories or generated in vitro after written informed consent using protocols approved by the Royal Free research ethics committee, Royal Free Hospital, London, UK. All animal work was performed under the authority of the UK Home Office Project and Personal Licenses regulations and was compliant with the guidelines of the University College London Ethics Committee.

3.1 Thawing and Passaging Human Fibroblasts

1. Warm all reagents and medium in a 37 °C water bath.
2. Obtain a frozen cryovial of patient fibroblasts (containing between 10^6 and 2×10^6 cells) from liquid nitrogen storage and keep on dry ice during transfer to the laboratory.
3. Transfer cryovial to a 37 °C water bath until approximately 30 % of ice crystals are present. Using a 1,000 μl pipette, transfer the entire thawed cell suspension to a 15 ml tube.
4. Immediately add 10 ml of pre-warmed fibroblast medium dropwise to the thawed cell suspension.
5. Centrifuge the mixture for 5 min at 400 × *g* and resuspend the pellet in 1,000 μl of fibroblast medium ensuring a single cell suspension. Add a further 11 ml of fibroblast medium and transfer to a T-75 flask.
6. Leave cells in the incubator overnight and refresh medium the next day to remove residual traces of DMSO.
7. After 3–5 days or once fibroblasts reach 80–90 % confluency, passage the cells once prior to reprogramming to ensure cells are in the growth phase.
8. To passage fibroblasts, aspirate medium and wash once with 10 ml of PBS. Aspirate and add 2 ml of trypLE express.
9. Leave in the incubator for 3–5 min and dislodge cells by gentle tapping. Add 4 ml of fibroblast medium and transfer to a 15 ml tube. Centrifuge for 5 min at 400 × *g*.
10. Resuspend the pellet in 1,000 μl of fibroblast medium ensuring a single cell suspension. Add a further 11 ml of fibroblast medium and transfer to a T-175 flask. Top up with another 10 ml of fibroblast medium and leave in the incubator. Refresh with 25 ml of fibroblast medium every second day.

3.2 Episomal Plasmid Reprogramming

1. Day 0: When fibroblasts reach 80 % confluency collect using TrypLE and wash once with PBS (*see* **Note 1**). Count viable cells using trypan blue and transfer 10^6 cells to a new tube. Centrifuge for 5 min at 400 × g. Remove supernatant and resuspend 10^6 cells in 100 μl of complete nucleofector solution R (Amaxa). Add 1 μl of each episomal plasmid (1 μg of each) to the cell suspension (*see* **Note 2**). Immediately transfect cells using program U-023 on a Nucleofector 2b device. Following transfection, resuspend cells in fibroblast medium and transfer them to a 10 cm tissue culture dish previously coated with 0.1 % gelatin (*see* **Note 3**).
2. From day 1 to day 7: change medium daily with fresh fibroblast medium supplemented with 0.5 mM sodium butyrate. On day 7 post transfection, count cells using trypan blue and seed 2×10^5 viable cells into one well of a 6-well plate coated with Geltrex, recombinant vitronectin, or Synthemax-R.

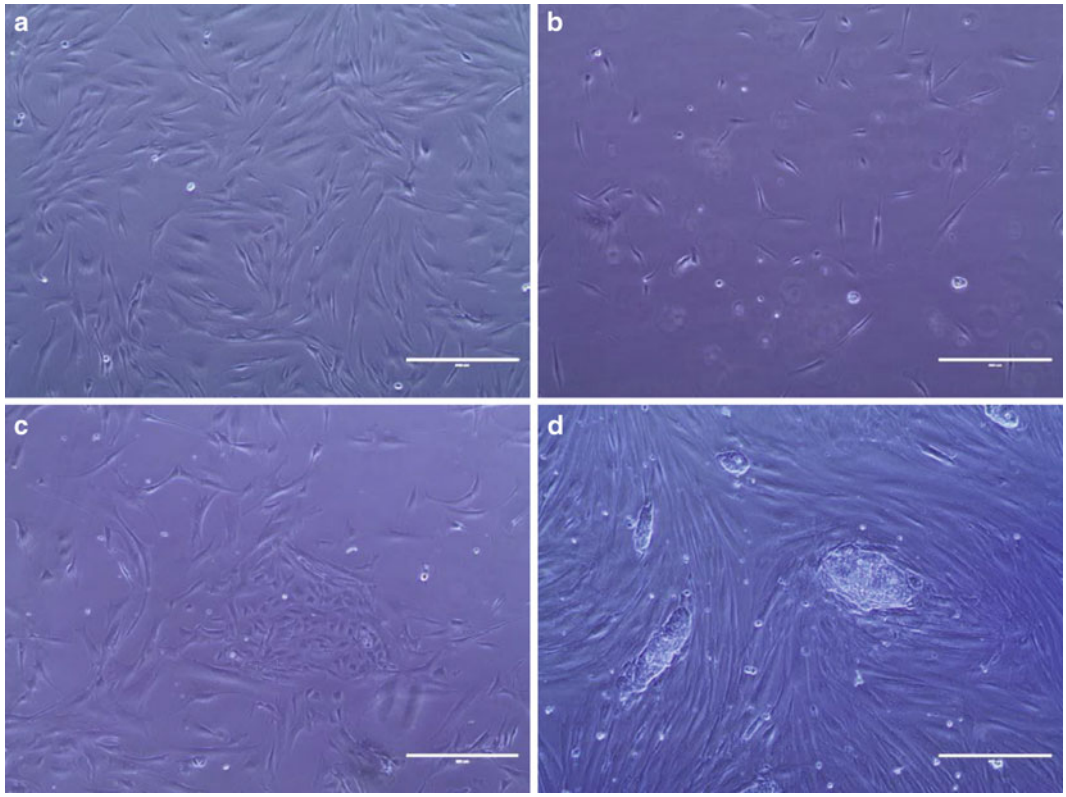

Fig. 1 Scale bar is 400 μm (**a**) Patient fibroblasts (Hermansky Pudlak Syndrome type 1, HPS1) at passage 7 at 60–70 % confluency. (**b**) Patient fibroblasts 1 day after transfection with reprogramming plasmids. (**c**) Patient fibroblasts 7 days after transfection with reprogramming plasmids, showing first signs of iPSC colony formation. (**d**) Patient fibroblasts 20 days after transfection with reprogramming plasmids, showing small iPSC colonies

3. From day 8 to day 11: change medium with fresh mTeSR1 or E8 medium supplemented with 0.5 mM sodium butyrate. Change medium daily.
4. From day 12: stop sodium butyrate treatment and change medium with fresh mTESR1 or E8 medium. Change medium daily until colonies form (around 30 days after transfection). *See* Fig. 1 for morphology changes of fibroblasts during reprogramming.
5. When colonies form, live stain cells with TRA-1-81 or TRA-1-60: dilute TRA-1-81 or TRA-1-60 primary antibody 1:100 and goat anti mouse IgG/IgM conjugated Alexa 488 secondary antibody 1:400 in pre-warmed DMEM/F12 and add to the cells. After 30 min, wash plates two times with DMEM/F12 and add fresh mTESR1/E8 medium.
6. Proceed to picking TRA-1-81 positive colonies under microscope using a fine tip plastic Pasteur pipette and transfer single

colonies to one well of a 12-well plate coated with Geltrex and containing 1 ml mTESR1 medium (10 μM of ROCK inhibitor can be added to aid attachment). Transfer a minimum of 12 putative iPSC colonies (*see* **Note 4**).

7. To calculate reprogramming efficiency, count the number of TRA-1-81 positive cells prior to picking them.

3.3 Maintenance and Cryopreservation of Human Pluripotent Stem Cells

1. Mechanically passage iPSCs into smaller clumps using the fine tip plastic Pasteur pipette under the stereomicroscope for the first three passages since initial derivation (*see* **Note 5**).
2. For subsequent passages (passage 3 onwards), use gentle cell dissociation reagent to passage cells (*see* **Note 6**). First, wash the iPSCs once with PBS, then add 1 ml per well (of a 6-well plate) of gentle cell dissociation reagent. Leave at room temperature for 5 min.
3. Aspirate the gentle cell dissociation reagent and add 1 ml of mTESR1 or E8 medium to the well. Use a cell lifter to scrape the well, so that all iPSCs are in suspension. Alternatively, a 5 ml shorty glass serological pipette can be used to scrape the cells.
4. Transfer cell suspension to a 15 ml centrifuge tube using a 5 ml serological pipette and gently pipette up and down 5–8 times to generate small clumps of iPSC colonies.
5. Transfer immediately to fresh Geltrex coated plates containing mTESR1 or E8 medium at the appropriate split ratios. (1:3 to 1:10 splits can be performed depending on growth characteristics of cells). Change medium daily.
6. For cryopreservation of iPSCs, pretreat iPSCs with 10 μM of ROCK inhibitor for 1–2 h prior to freezing. We routinely freeze 1 well of a 6-well plate to 1 cryovial.
7. Harvest iPSCs as above using gentle cell dissociation reagent but collect cell suspension in a 15 ml tube.
8. Centrifuge cells for 5 min at 200 × *g*. Resuspend the pellet in 50 μl of mTESR1 or E8 medium being careful not to create a single cell suspension. Transfer this cell suspension to one cryovial. To this add 1 ml of Bambanker solution. Re-cap the cryovial and gently flick the tube to ensure cells are suspended. Leave in a −80 °C freezer overnight in a Mr Frosty.
9. Immediately transfer to liquid nitrogen storage facilities the next day.

3.4 Characterization of Human Pluripotent Stem Cells

3.4.1 Immunostaining

1. Culture iPS cells on Geltrex coated plates. Aspirate medium, wash cells with PBS and then fix them with 4 % paraformaldehyde (PFA) for 20 min (*see* **Note 7**).
2. Remove PFA and wash cells 3× with PBS and either store them at 4 °C in PBS or directly use them for immunostaining.

3. If performing nuclear staining (for intracellular antigens such as Oct4), you need to permeabilize the cells with ice-cold methanol (keep methanol in −20 °C before use) for 5 min prior to blocking. Remove methanol and wash 1× with PBS.
4. To block cells add blocking solution for 30 min–1 h. Remove blocking solution and add primary antibody diluted 1:100 in blocking solution (OCT3/4, TRA-1-60, TRA-1-81, SSEA-1, SSEA-3, and SSEA-4) for 1 h at room temperature or overnight at 4 °C.
5. Wash off primary antibodies by rinsing cells 3× with PBS and incubate cells with appropriate secondary antibodies (Alexa 488 anti-mouse—Life Technologies) at 1:400 dilution in blocking solution for 1 h at room temperature covered from light.
6. Wash off secondary antibodies by rinsing cells 3× with PBS. Add nuclear stain (DAPI or PI) and incubate for 5 min at room temperature covered from light.
7. Wash cells by rinsing cells 3× with PBS. Add PBS to the cells and proceed to imagining cells immediately. Human iPSCs should be negative for SSEA-1 but positive for Oct3/4, TRA-1-60, TRA-1-81, SSEA-3, and SSEA-4. *See* Fig. 2 for images of immunostained patient specific iPS lines.

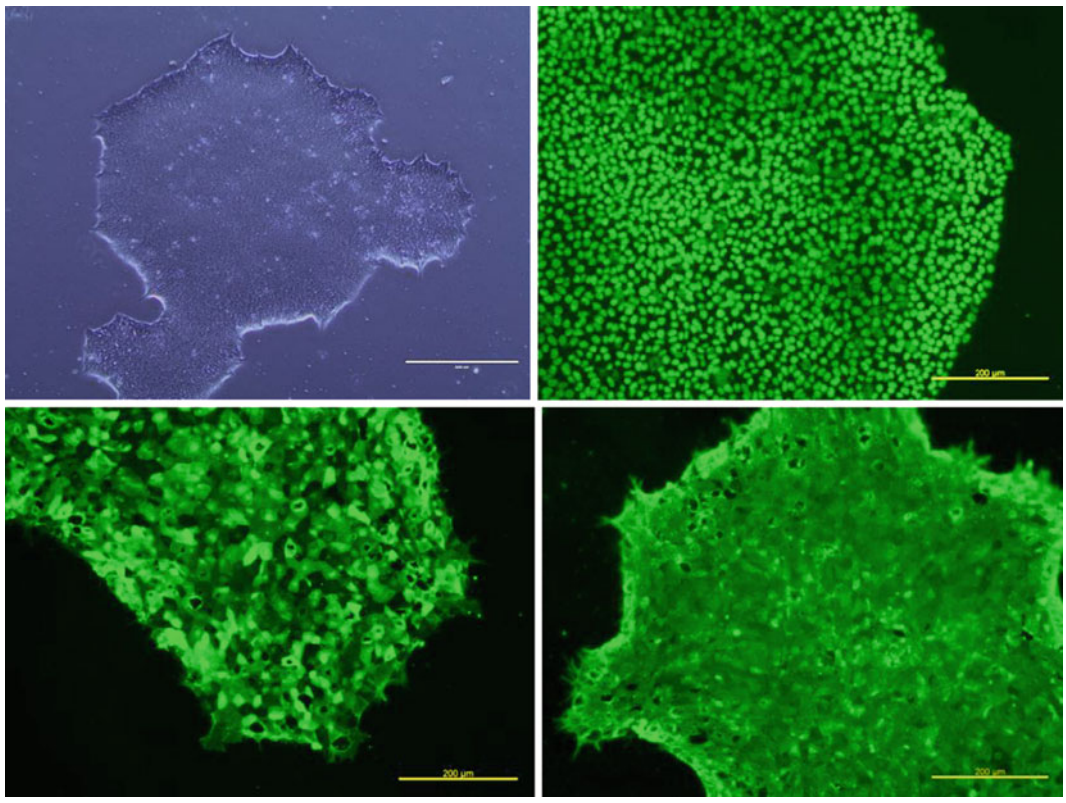

Fig. 2 Scale bar is 200 μm (**a**) Patient specific iPSC line (HPS1) at passage 22. (**b**) Oct4 immunostaining (**c**) TRA-1-81 immunostaining (**d**) SSEA-4 immunostaining

3.4.2 QRT-PCR

1. Isolate RNA from cells using TRIzol method: Add 1 ml TRIzol to 5×10^6 iPS cells. Lyse cells in sample by pipetting up and down several times. Add 0.2 ml chloroform for each 1 ml of TRIzol used and vigorously shake the tube for 15 s. After 2–3 min at room temperature, centrifuge sample at 12,000 × *g* for 15 min at 4 °C. Remove the aqueous phase of the sample by angling the tube and pipetting the solution out. Avoid drawing any of the interphase or organic layer in the pipette. Place the aqueous phase into a new tube and add 0.5 ml of 100 % isopropanol per 1 ml of TRIzol used. Incubate at room temperature for 10 min and centrifuge at 12,000 × *g* for 10 min at 4 °C. Remove supernatant and wash pellet with 1 ml of 75 % ethanol per 1 ml TRIzol used in the initial homogenization. Vortex sample briefly and centrifuge the tube at 7,500 × *g* for 5 min at 4 °C. Discard wash and air-dry pellet. Resuspend RNA in RNase-free water by pipetting the solution up and down several times. Incubate at 55–60 °C for 10–15 min.
2. Measure the amount and purity of extracted RNA by absorbance at 260/280 nm on a NanoDrop Spectrophotometer. If not used immediately, store RNA at −80 °C.
3. For cDNA conversion, first treat 2 μg of RNA with DNase before conversion using Superscript III according to manufacturer's instructions. Store cDNA at −20 °C and use 5 μl for subsequent PCR reactions.
4. Use Sybr Green according to manufacturer's instructions for RT-PCR reactions (*see* **Note 8**).

3.4.3 Flow Cytometry

1. Aspirate medium from wells of a 6-well plate and wash cells twice with PBS.
2. Add 1 ml of pre-warmed Accutase and incubate cells at 37 °C for approximately 5 min until cell clumps dissociate.
3. Gently resuspend the cells in 1 ml mTESR1.
4. Transfer cells to a 15 ml tube and centrifuge at 200 × *g* for 5 min.
5. Resuspend 1×10^5 cells in 200 μl FACS buffer for each sample.
6. Add 2 μl of primary antibody and incubate for 1 h at 4 °C in the dark.
7. Add 0.2 μl of secondary antibody and incubate for an additional hour at 4 °C in the dark.
8. Wash the cells with 3 ml FACS buffer (200 × *g* for 5 min).
9. Resuspend cells in 500 μl FACS buffer.
10. Analyze stained samples by flow cytometry, using unlabelled cells and cells with secondary antibody-only controls.

3.4.4 Genomic PCR for Detecting the Integration of Plasmids

1. Perform DNA extraction using the DNeasy blood and tissue kit (Qiagen) according to manufacturer's instructions and store genomic DNA at −20 °C. Use plasmid constructs and day 7 transfected fibroblasts as positive controls.
2. Measure the amount and purity of extracted DNA by absorbance at 260/280 nM on a NanoDrop Spectrophotometer.
3. Use GoTaq green mastermix with 100–200 ng of DNA according to manufacturer's instructions for genomic PCR reactions. Use a total of 30 amplification cycles of denaturation at 94 °C for 30 s, annealing at 55–58 °C for 30 s and extension at 72 °C for 1 min. Include a first denaturation step at 94 °C for 5 min and a final extension step at 72 °C for 5 min. Primer sequences have been previously described [17].
4. Run PCR products on a 1 % agarose gel at 80–100 V for 2 h.

3.4.5 Teratoma Formation

1. Pretreat 1 × 6 well plate of confluent iPSCs with 10 μM of ROCK inhibitor for 1–2 h prior to use.
2. Harvest iPSCs as per Section 3.3 and resuspend the cell pellet in 70 μl of mTESR1 medium and add 30 μl of undiluted Geltrex. Transport iPSC on ice.
3. Anesthetize one 6–8-week-old male NOD-SCID mouse by isoflurane inhalation and inject 50 μl of the iPSC suspension into each testis capsule. Administer analgesia (Carprofen, 5 mg/kg) intraperitoneally following surgery to minimize pain.
4. After 8–12 weeks or when animals appear to have a bloated stomach, sacrifice animals and excise and fix teratomas in Histochoice (Amresco).
5. Send fixed tissue to a histology facility for embedding, cutting and haematoxylin and eosin (H&E) staining. Advice of a certified pathologist is required to ascertain the presence of tissue derivatives indicative of the three germ lineages.

4 Notes

1. The quality and condition of fibroblasts is vital for efficient reprogramming. Fibroblasts must be actively proliferating and best used before passage 12.
2. Have reprogramming plasmids at the same concentration of 1 μg/ml to avoid diluting the nucleofector solution and avoid pipetting errors.
3. GFP expressing plasmid (pCXLE-EGFP) is included in parallel experiments to measure the transduction efficiency of fibroblasts. We routinely obtain >40 % GFP transfection efficiency

of fibroblasts as measured by flow cytometry using the 2b machine. If the transfection efficiency is below this, then the efficiency will be low.

4. Not all putative iPSC colonies can be established into stable iPS lines, as some are only partially reprogrammed. Therefore, several colonies need to be expanded initially. By passage 5–6, only the iPS lines showing the best morphology need to be expanded.
5. Do not let iPS colonies grow so large that the edges of different colonies begin to touch each other as this will result in differentiation and poor survival/attachment after passaging. ROCK inhibitor (10 μM) can be added to the cultures for 24 hours after passaging to improve survival.
6. It is possible to use dispase (1 mg/ml) for passaging instead of gentle dissociation reagent but in our experience, higher split ratios can be performed with the gentle dissociation reagent.
7. PFA is toxic. Perform experiments in a fume cupboard and follow local rules for disposal.
8. Perform gene expression analysis using the DELTA CT method. GAPDH used as endogenous control.

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