



Kejin Hu *Editor*

Nuclear Reprogramming

Methods and Protocols



METHODS IN MOLECULAR BIOLOGY

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Nuclear Reprogramming

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Edited by

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Preface

Nuclear reprogramming is a science. As a science, it was established when Sr. John Gurdon cloned the first animal, frog, more than 60 years ago. His achievement proved that a somatic cell harbors all the genetic materials as do the totipotent cells at the early stage of development.

Nuclear reprogramming is more an art in practice. Mastery of this art determines the quality and levels of the work. Human beings only mastered the skills of nuclear reprogramming to the level capable of cloning the first mammals almost 40 years after Gurdon cloned the frogs. It took additional more than 20 years of practice for us to be able to clone monkeys using the same genre of techniques. In 2006, we have expanded our skill set in nuclear reprogramming when Dr. Yamanaka developed a simple method to reprogram somatic cells into pluripotency. His group reported that fibroblasts can be induced to pluripotent stem cells (iPSCs) simply by forced expression of only four genes. This technique has extensively expanded the field of nuclear reprogramming, and now we can generate many types of cells by factor reprogramming of another unrelated type of cells. Nowadays, the art of nuclear reprogramming is no longer the exclusive skills of a small group of the elite scientists; yet, nuclear reprogramming is still challenging for even the experienced laboratories.

To become an artist, one needs tutors and/or tutorials. I was very embarrassed when I could not draw a decent mouse on the white board in the class of the Advanced Stem Cell Biology and Regenerative Medicine I was teaching. This is not because I do not have the genetic components for drawing. The talent of my 13-year-old son tells me that I have the potential to draw a great mouse. You will see that when you compare the camera-generated picture of mine and my portrait sketched by my 13-year-old boy at the end of this introduction. I just did not have a tutor and tutorials. I even did not have pencils and papers in my house when I was young.

To become a great artist in nuclear reprogramming, one also needs tutors and tutorials during their practices. This volume serves for this purpose. It compiles a set of detailed protocols for various types of nuclear reprogramming including animal cloning using somatic cell nuclear transfer (SCNT), as well as neural, cardiac, and pluripotency reprogramming using various technologies including protein transduction, episomal plasmids, lentiviral vectors, gamma retroviral vectors, mRNA transfection, microRNA transfection, and of course the latest CRISPR technology.

Pig is not only an essential farm animal, but also serves as critical models in medical research, and vehicles in biotechnologies. The laboratories of Drs. Ouyang, and Han jointly provide a detailed protocol about pig cloning using the technique of somatic cell nuclear transfer (SCNT) (Chapter 1). The recent successful cloning of monkey represents a new level of the nuclear reprogramming art, and Dr. Sun's group in Chapter 2 provides us with step-by-step procedures for cloning of monkey via SCNT.

Factor-induced cardiomyocytes open up a new avenue for studying cardiac diseases and also hold promise for heart regeneration. Dr. Fu's laboratory in Chapter 3 describes procedures to generate human cardiomyocytes by reprogramming of fibroblasts via ectopic expression of lineage-specific transcription factors while Dr. Dzau's group in Chapter 4 describes similar work but using microRNA to reprogram.

Neural reprogramming of somatic cells is a major interest in the nuclear reprogramming field. Chapter 5 details a technology to generate transgene-free human neural progenitors from fibroblasts using a suicide gene on the episomal vectors. In Chapter 6, Dr. Yoo's group

provides detailed protocol about reprogramming of human fibroblasts into neurons using microRNA-9/9^{*} and microRNA-124.

Reprogramming of human fibroblasts using lentiviral vectors remains as the key technology in studying the molecular mechanisms of nuclear reprogramming because of its efficiency and reliability. In Chapter 7, my laboratory describes the detailed procedures for lentiviral reprogramming of human fibroblasts into the pluripotent state.

For iPSCs to be used in the clinical setting, it is critical to generate human iPSCs without transgene footprints. In the following chapters, various technologies to generate the transgene-free human iPSCs are detailed including reprogramming with episomal plasmids (Chapters 8 and 9), proteins (Chapter 10), and mRNA (Chapter 11).

A new tool in the nuclear reprogramming toolkit box is CRISPR technology. In Chapter 12, Drs. Weltner and Trokovic describe how to activate the endogenous pluripotency regulatory networks using the CRISPR-mediated activator domains, and eventually generate iPSCs.

One challenging in pluripotent stem cell technology is that it is difficult to establish bona fide stable pluripotent stem cell lines for the large animals. Recently, this field made a great progress by generating extended potential stem cells (EPSCs). In Chapter 13, Dr. Liu and colleagues provide the detailed technical parts of their recent achievement.

In addition to techniques for nuclear reprogramming, this volume also compiles several chapters that describe the related technologies including preparation of mouse embryonic fibroblasts (MEF) to support reprogramming and maintenance of the generated iPSCs by the WiCell experts (Chapter 14), common characterization of the generated human iPSCs (Chapter 15), and karyotyping of the resulting iPSC lines (Chapter 16).

Lastly, I am very happy to contribute a chapter by myself. This chapter can fit any volume of the MiMB series because it will help most, if not all, of the biomedical laboratories. In Chapter 17, I provide a step-by-step tutorial for a novice without any prior R experience to prepare professional heat maps in the R platform in just one day. Positive feedbacks from my friends encouraged me to include this tutorial in this volume. I proudly envision that this tutorial will be used the most among all the protocols included here although my chapter has no direct scientific significance.

I hope this volume will be the handbook for many laboratories in the field of nuclear reprogramming.

Birmingham, AL, USA

Kejin Hu

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Chapter 1

Pig Cloning Using Somatic Cell Nuclear Transfer

Hongsheng Ouyang, Jianyong Han, and Yongye Huang

Abstract

Porcine cloning technology can be used to produce progenies genetically identical to the donor cells from high-quality breeding pigs. In addition, genetically modified pigs have been produced by somatic cell nuclear transfer using genetically modified porcine fetal fibroblasts. The method of preparing genetically modified pigs is critical for establishing pig models for human diseases, and for generating donor animals for future xenotransplantation. This chapter describes detailed procedures for generating cloned pigs using fetal fibroblasts as nuclear donors.

Key words Pig cloning, Somatic cell nuclear transfer, Animal cloning, Oocytes, Fetal fibroblasts

1 Introduction

The first cloned pigs based on somatic cell nuclear transfer (SCNT) were generated in 2000 [1, 2]. Porcine cloning technology can be used to produce progenies genetically identical to the donor cells from the existing high-quality breeding pigs. In addition, many valuable biomedical and agriculture swine models have been produced by somatic cell nuclear transfer using genetically modified porcine fetal fibroblasts [3–5]. For example, pigs resistant to the classical swine fever viruses and those for Huntington’s disease models have been generated by SCNT [6, 7].

At present, the most frequently used nuclear donor cells for cloning pigs are fibroblasts isolated from pig fetuses of 35–40 days [8]. The developmental competence of cloned porcine embryos using adult fibroblasts as nuclear donors is lower than that using fetal fibroblasts [9]. Treatments of donor cells with *xenopus* egg extracts, 5-aza-dC, TSA, or aphidicolin improve the development of the cloned porcine embryos [10–12]. In addition to fibroblasts, cumulus, mesenchymal stem cells, preadipocytes, oviduct cells, kidney cells, and liver cells have been used as nuclear donors for porcine SCNT [9, 13–16].

The oocytes required for the generation of cloned porcine embryos are mainly collected from the slaughter sow ovary. The size of the follicles used to collect porcine oocytes affects the in vitro maturation of oocytes and the development potential of the cloned embryos. Oocytes collected from follicles with a diameter of 3–8 mm have better in vitro developmental potential than those collected from follicles with a diameter of less than 3 mm. As a result, larger oocytes collected from 3–8 mm follicles have higher cloning capacity than smaller ones [17].

The composition of in vitro maturation media for porcine oocytes also affects the maturation of oocytes and the developmental competence of the SCNT embryos. Many components of the in vitro oocyte maturation medium have been reported to benefit the development of the SCNT embryos including leptin, insulin-transferrin-selenium, dibutyryl cyclic AMP, L-carnitine, resveratrol-melatonin supplementation, MC1568, cilostamide, alpha-linolenic acid, canthaxanthin, zinc, cilostazol, lanosterol, and colcemid [18–30]. When subjected to SCNT, embryos derived from the sexually mature sow oocytes developed to blastocysts at higher rates and had higher cell number than those derived from the immature gilt oocytes [31, 32]. The in vitro maturation medium for meiotic maturation of oocytes supplemented with three cytokines (FGF2, LIF, and IGF1) improves nuclear maturation of the oocytes in cumulus-oocyte complexes derived from the immature pig ovaries [33].

The enucleated porcine oocyte can be electrically fused with the donor cell and activated simultaneously. Adding certain chemical additives (6-DMAP or AZD5438) during the electrical activation can enhance the fusion rate between porcine oocytes and donor cells, as well as improve the developmental competence of the cloned porcine embryos [34, 35]. The matured oocytes could be activated by the treatment with ionomycin and TPEN [36]. The cloning efficiency can be enhanced by treatments of the reconstructed porcine embryos with the histone deacetylase inhibitor (trichostatin A, scriptaid, valproic acid, sodium butyrate, oxamflatin, panobinostat, CUDC-101, *m*-carboxycinnamic acid bishydroxamide, suberoylanilide hydroxamic acid, M344, LAQ824, quisinostat, MGCD0103, abexinostat, suberoylanilide hydroxamic acid, 4-iodo-suberoylanilide hydroxamic acid, belinostat, LBH589 or CI994), and other molecules including alpha-tocopherol, L-ascorbic acid, insulin-like growth factor-I, phytohemagglutinin, MG132, latrunculin A, 5-aza-2'-deoxycytidine, melatonin, EPZ004777, demecolcine and 6-dimethylaminopurine, BIX-01294, taurooursodeoxycholic acid, RepSox, RG108, and chaetocin [37–68]. In general, pig embryos established by SCNT are transferred at the one-cell stage because of the suboptimal culture conditions for embryos currently available. Pig SCNT-derived blastocysts cultured in vitro with GM-CSF can successfully

produce piglets [69]. In this chapter, we describe the detailed procedures for generation of cloned pigs by SCNT using the fetal fibroblasts as the nuclear donor.

2 Materials

2.1 Equipment, Instruments, Disposable Plasticware

1. Biosafety cabinet for cell culture.
2. Centrifuge, for cell culture.
3. Incubator(s), humidified, at 38.5 °C, 5% CO₂, for embryo and cell culture.
4. Inverted microscope, with phase contrast for cell culture observations.
5. Stereo microscope, for embryo and oocyte culture observations.
6. Micromanipulators.
7. Sachs-flaming micropipette puller: for making enucleation and injection pipet.
8. Microforge: for making enucleation and injection pipet.
9. Grinding wheel: for making injection pipet.
10. Coagulation tubes: for enucleation and injection.
11. Petri dishes, various sizes for cell culture and embryo manipulations.
12. PCR Cycler.
13. Nalgene Mr. Frosty Freezing Container.
14. Cell transportation box.
15. Cell electrofusion device (BTX ECM 2001).
16. Anesthesia machine.
17. Catheter, open-end catheter with adapter, 11.4 cm.

2.2 Reagents and Solutions

1. Dissection buffer: DPBS without calcium and magnesium.
2. DMEM.
3. 10× collagenase stock: DMEM supplemented with 20% fetal bovine serum (FBS), 2000 units/mL collagenase, 250 units/mL DNase.
4. 1× digestion medium: Add 10 mL of 10× collagenase stock into 90 mL of DMEM and mix.
5. Any quality genomic DNA kit (we used TIANamp).
6. 2× Taq Plus PCR MasterMix (TIANGEN, KT205 or any of your choices).

7. Sex identification primers: 10 μ M SRY-F: GCT TTC ATT GTG TGG TCT CGT, 10 μ M SRY-R: CTT GGC GAC TGT GTA TGT GAA G, 10 μ M GAPDH-F: GAT GGC CCC TCT GGG AAA CTG TG, 10 μ M GAPDH-R: GGA CGC CTG CTT CAC CAC CTT CT.
8. Agarose.
9. Cell culture medium: DMEM supplemented with 20% fetal bovine serum.
10. Cryopreservation medium: 10% DMSO in FBS.
11. 1 \times PVA-TL HEPES stock: 0.1 g/L polyvinyl alcohol (PVA), 6.663 g/L NaCl, 0.237 g/L KCl, 0.041 g/L NaH₂PO₄, 1.868 mL/L Na Lactate, 2.383 g/L HEPES, 0.065 g/L penicillin G, 2.186 g/L sorbitol, 0.294 g/L CaCl₂ \cdot 2H₂O, 0.102 g/L MgCl₂ \cdot 6H₂O, 0.025 g/L gentamicin, 0.06 g/L streptomycin, 0.022 g/L sodium pyruvate, 0.168 g/L NaHCO₃, 0.01 g/L phenol red. Adjust the pH to 7.2–7.4 with HCl and the osmolarity to 295–310 mOsm using NaCl.
12. Basic medium: 1 g/L PVA, 0.55 g/L d-glucose, 0.1 g/L sodium pyruvate, 0.075 g/L penicillin, 0.05 g/L streptomycin, 9.5 g/L TCM-199.
13. 100 \times cysteine stock: 57 mM cysteine.
14. 100 \times LH stock: 50 μ g/mL luteinizing hormone (LH).
15. 100 \times FSH stock: 50 μ g/mL follicle-stimulating hormone (FSH).
16. 1000 \times EGF stock: 10 μ g/mL epidermal growth factor (EGF).
17. Oocyte maturation medium: 6952 μ L basic medium, 800 μ L follicular fluid, 80 μ L 100 \times cysteine stock, 80 μ L 100 \times LH stock, 80 μ L 100 \times FSH stock, 8 μ L 1000 \times EGF stock.
18. Oocyte manipulation medium: 1.755 g/L NaCl, 0.75 g/L HEPES, 0.05 g/L penicillin, 0.06 g/L streptomycin, 0.05 g/L NaHCO₃, 9.5 g/L TCM-199, 3 g/L BSA. Adjust the pH to 7.2–7.4 with NaOH and the osmolarity to 295–310 mOsm using NaCl.
19. Cytochalasin B stock: 1 mg of cytochalasin B in 200 μ L of 100% ethanol.
20. Enucleation medium: 4 μ L cytochalasin B stock and 3 mL oocyte manipulation medium.
21. Oocyte-denuding medium: 100 mg hyaluronidase, 6 g mannitol, 0.001 g BSA, 5 mL PVA-TL HEPES stock, and 95 mL Milli-Q H₂O. Adjust the pH to 7.0–7.4 with HCl and NaOH. Prepare 1-mL aliquots in 1.5 mL microfuge tubes and store the aliquots in the –20 °C freezer.

22. 0.25% trypsin: 0.25 g trypsin and 100 mL PBS, adjust pH to 7.4.
23. Fusion medium: 54.651 g/L mannitol, 0.147 g/L CaCl₂·2H₂O, 0.02 g/L MgCl₂·6H₂O, 0.13 g/L HEPES. Adjust the pH to 7.0–7.4.
24. Porcine zygote medium-3 with BSA (PZM-3 with BSA) 6.312 g/L NaCl, 0.746 g/L KCl, 0.048 g/L KH₂PO₄, 0.146 g/L L-glutamine, 0.086 g/L MgSO₄·7H₂O, 0.616 g/L Ca-lactate·5H₂O, 0.546 g/L Hypotaurine, 0.022 g/L Na-pyruvate, 0.066 g/L penicillin, 0.050 g/L streptomycin, 20 mL/L BME amino acid solution, 10 mL/L MEM non-essential amino acid solution (NEAA), 2.106 g/L NaHCO₃, 3 g/L BSA, adjust pH to 7.2–7.4 and the osmolarity to 286–290 mOsm.
25. Isoflurane.
26. Propofol: 10 g/L propofol.
27. 1000× IGF1 stock: 15 µg/mL insulin-like growth factor 1 (IGF1).
28. 1000× LIF stock: 15 µg/mL leukemia inhibitory factor (LIF).
29. 1000× FGF2 stock: 25 µg/mL fibroblast growth factor 2 (FGF2).
30. Oocyte maturation medium-1: 6928 µL basic medium, 800 µL follicular fluid, 80 µL 100× cysteine stock, 80 µL 100× LH stock, 80 µL 100× FSH stock, 8 µL 1000× EGF stock, 8 µL 1000× IGF1 stock, 8 µL 1000× LIF stock, 8 µL 1000× FGF2 stock.

3 Methods

3.1 Isolation of Porcine Fetal Fibroblasts

1. Anesthetize a sow at 30–34 days of gestation. Remove the uterus by surgery, and put the uterus in a sterile box at 4–10 °C for transportation. Transport the uterus to the laboratory within 4 h (*see Note 1*).
2. Strip the fetuses from the uterus and transfer each fetus into a sterile glass Petri dish (diameter: 100 mm).
3. Wash the fetuses with dissection buffer until blood-free.
4. Remove the head, limbs and viscera, and store them at –20 °C for sex identification and other analyses at later stages. Wash the remaining fetal tissues with dissection buffer.
5. Transfer the remaining fetal tissues into another sterile glass Petri dish (diameter: 100 mm), and cut the fetus into pieces of 1 mm³ with a pair of scissors.

6. Add 15–20 mL of digestion medium to the Petri dish, and incubate at 38.5 °C, 5% CO₂ in a humidified incubator for 2–6 h.
7. Transfer the digestion medium with the fetal pieces into a 50-mL centrifuge tube, and centrifuge at 500 × g for 5 min.
8. Remove the supernatant, resuspend the cell pellet in 10 mL DMEM + 5% FBS, and centrifuge at 500 × g for 5 min.
9. Remove the supernatant and resuspend the cell pellet in 10 mL DMEM + 20% FBS.
10. Transfer the cell suspensions into four dishes (100 mm in diameter).
11. Place the dishes in a CO₂ incubator (38.5 °C, 5% CO₂, 100% humidity) and culture until 80–90% confluence.

3.2 Cryopreservation of Porcine Fetal Fibroblasts

1. When the cells grow into 80–90% confluence, remove the culture medium and wash the cells twice using Ca²⁺ and Mg²⁺-free PBS.
2. Add 1 mL 0.25% trypsin into each dish and incubate at 38.5 °C for 1 min, and observe the cell morphology change under microscope. When the cells become round in shape, add 10 mL of culture medium to terminate digestion.
3. Pipet the culture medium gently to detach the cells from dish and transfer the cell suspension into a 15-mL centrifuge tube.
4. Centrifugation is performed at 100 × g, 10 min.
5. Remove the supernatant and resuspend cells using 3–4 mL freezing medium (10% DMSO and 90% FBS). The cell concentration in freezing medium is about 10⁷ cells/mL.
6. Transfer the cells into freezing tubes (1 mL/tube).
7. Place these tubes into a freezing box and keep the freezing box in –80 °C overnight.
8. The next morning, transfer these tubes from –80 °C into liquid nitrogen for long-term storage.

3.3 Sex Identification of Porcine Fetal Fibroblasts

To conduct sex identification, SRY gene is amplified via PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as an internal control for PCR amplification. The genomic DNA from male piglets gives a 309-bp band for the SRY amplicon, and a 404-bp band for the GAPDH amplicon on the agarose gel after electrophoresis while the PCR products for the female DNA have the GAPDH amplicon, but not the SRY amplicon.

1. Collect 10–30 mg fetus tissue using sterile scissors. Label the tissue using the same number with its corresponding fibroblast cells.
2. Add the collected tissue into a 1.5 mL microcentrifuge tube.

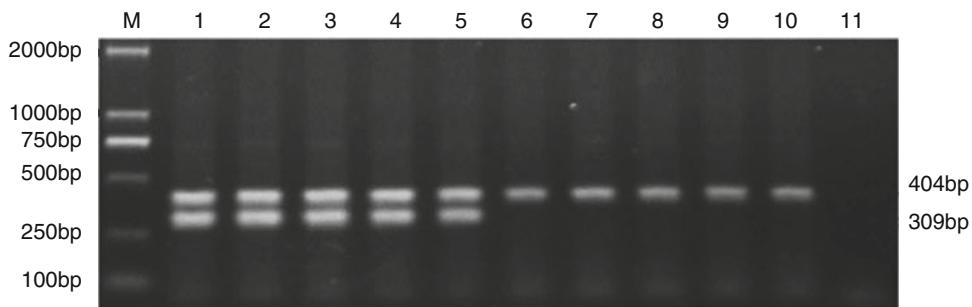


Fig. 1 Sex identification of porcine fetal fibroblasts. Lanes 1–5 represent the male fetal samples. Lanes 6–10 represent the female fetal sample. Lane 11, blank control. M, DNA2000 marker

3. Extract the genomic DNA using any quality genomic DNA kit (We used TIANamp, DP304).
4. Use 50 ng DNA as template for PCR amplification of the SRY gene: 50 ng template DNA, 1 μ L primer, 10 μ L 2 \times Taq Plus PCR Master Mix, and add H₂O to a final volume of 20 μ L.
5. The amplification program was as follows: initial denaturing at 94 °C for 3 min, followed by 30 cycles of (94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min). Carry out a final cycle of extension step at 72 °C for 5 min.
6. Assess the PCR products (10 μ L) by electrophoresis on a 2% agarose gel. Determine the sex based on the presence/absence of the SRY amplicon (Fig. 1).

3.4 Isolation and Culture of Porcine Oocytes

1. About 6 h before porcine ovaries collection, add 2 mL oocyte maturation medium into each of four culture dishes (30 mm in diameter). Then, cover the oocyte maturation medium with 1.5 mL of paraffin oil and transfer these dishes into a CO₂ incubator to equilibrate the medium (38.5 °C, 5% CO₂, 100% humidity).
2. Collect porcine ovaries without cysts from a slaughterhouse nearby and transport the ovaries to the laboratory in a thermo container filled with sterile saline water at 30–35 °C within 4 h after collection.
3. Upon arriving at laboratory, immediately wash the ovaries 3–4 times with saline and then transfer the ovaries into a 1-L beaker.
4. Place the beaker in a 38.5 °C water bath.
5. Aspirate the follicular contents from 3- to 8-mm follicles using 10 mL syringe (the needle 30° down into the follicle) and transfer the liquid into a 50-mL centrifuge tube.
6. After 5–10 min of sedimentation, remove the supernatant from the 50 mL centrifuge tube.

7. Add 7–8 mL of 1× PVA-TL HEPES stock into a 100-mm culture dish and place the dish on a 38.5 °C constant temperature heating plate. Transfer the sediment in the 50 mL centrifuge tube into a 100-mm culture dish evenly using a 1-mL pipette.
8. Select cumulus-oocyte complexes (COCs) with at least three uniform layers of cumulus cells using a sterile glass pipette and transfer them into a 30-mm culture dish filled with 1× PVA-TL HEPES stock.
9. Wash the COCs twice with 1× PVA-TL HEPES stock and then transfer the COCs into a 30-mm culture dish containing 2 mL of the oocyte maturation medium, which has been underwent pre-equilibrium in 38.5 °C CO₂ incubator in **step 1** above.
10. Record the number of the COCs and wash them twice in oocyte maturation medium.
11. Transfer the COCs into a 30-mm culture dish containing 2 mL of oocyte maturation medium and mature the oocytes for 42–44 h in a CO₂ incubator (38.5 °C, 5% CO₂, 100% humidity) (*see Note 2*).

3.5 Denudation of Porcine Oocytes

1. Add 2 mL oocyte manipulation medium into each of four culture dishes (30 mm in diameter). Then, cover the oocyte manipulation medium with 1.5 mL paraffin oil.
2. Make 7 individual drops of oocyte manipulation medium in a 60-mm culture dish. Each drop is around 100 µL. Cover the entire dish with 7 mL of paraffin oil.
3. Add 2 mL of oocyte manipulation medium and 4 µL cytochalasin B stock into a 30-mm culture dish (named enucleation medium).
4. Make 3 individual drops of 100 µL enucleation medium in a 30-mm culture dish and cover the entire dish with 2 mL paraffin oil.
5. Transfer the above dishes in **steps 1, 2, and 4** onto a 38.5 °C constant temperature heating plate.
6. Transfer the COCs which have been matured for 42–44 h from the CO₂ incubator onto the 38.5 °C constant temperature heating plate.
7. Transfer the above COCs into a microtube (1.5 mL) containing oocyte-denuding medium and pipet 60–90 times.
8. Transfer the above COCs into a 30-mm culture dish containing 2 mL of the oocyte manipulation medium as prepared in **step 1**.
9. Pick up the denuded oocytes into another 30-mm culture dish containing 2 mL of oocyte manipulation medium as prepared in **step 1**.

10. Select oocytes that have an intact plasma membrane, round shape, and visible perivitelline space and transfer them into another 30-mm culture dish containing 2 mL of oocyte manipulation medium.
11. Transfer the above oocytes into another 30-mm culture dish containing 2 mL of oocyte manipulation medium.
12. Pick up the denuded oocytes that have polar bodies and transfer them into the 60-mm culture dishes with 7 drops of 100 μ L oocyte manipulation medium as prepared in **step 2**.

3.6 Digestion of Porcine Fetal Fibroblasts

The cells used for nuclear transfer (i.e., porcine fetal fibroblast) are cultured in a 24-well plate and collected by trypsinization treatment (*see Note 3*).

1. Remove the culture medium from the well and wash the cells twice using pre-warmed PBS at 38.5 °C.
2. Add 200 μ L 0.25% trypsin into each well and incubate at 38.5 °C for 1 min. Observe the cell morphology change under microscope. When the cells become round in shape, add 1 mL culture medium to terminate digestion.
3. Pipet the culture medium gently to detach the cells from the plate and transfer the cell suspension into a 1.5-mL centrifuge tube.
4. Centrifuge the cells at $100 \times g$ for 10 min. Remove the supernatant.
5. Add 1 mL of the oocyte manipulation medium into the tube and pipet several times.
6. Centrifuge the cells at $100 \times g$ for 10 min. Remove the supernatant.
7. Add 100 μ L enucleation medium into the tube and pipet several times.

3.7 Nuclear Transfer

1. Turn on the power key of the micromanipulator and turn on the hot plate. Adjust the temperature of the hot plate to 38.5 °C.
2. Make 10 individual drops of 6 μ L enucleation medium in the cover of a 60-mm culture dish (4 drops in the middle, 3 drops each on the left and right sides). Cover the entire dish with 7 mL of paraffin oil. Place the cover onto a 38.5 °C constant temperature heating plate.
3. Wash the oocytes using enucleation medium three times.
4. Transfer the above oocytes into the 6- μ L drops in **step 2** (30–50 oocytes per drop). Place about 0.5- μ L fetal fibroblasts around the oocytes.
5. Place this cover onto the heat plate in the micromanipulator.

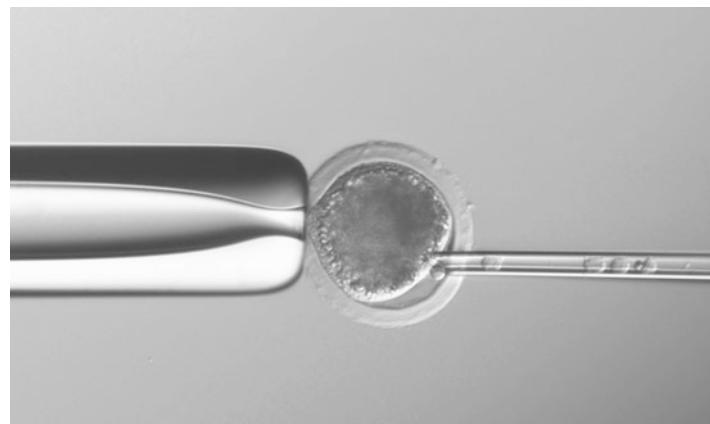
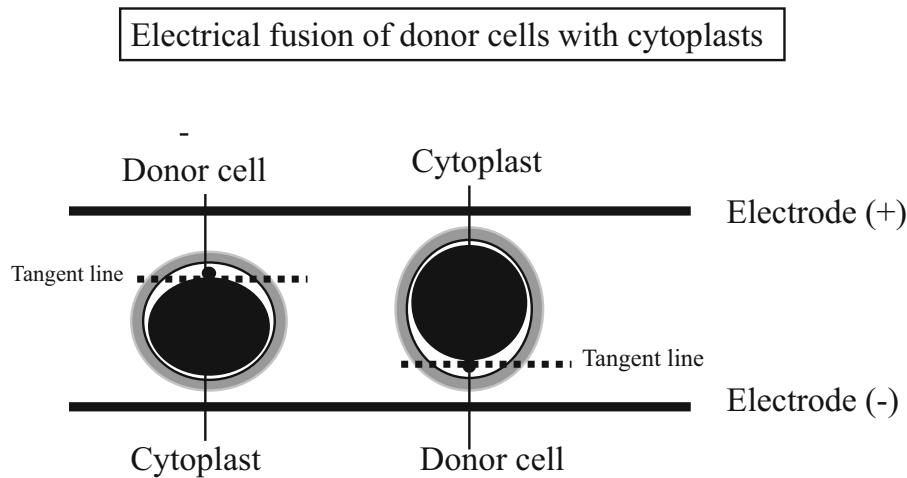


Fig. 2 Performing somatic cell nuclear transfer. An oocyte is held by the oocyte holding needle (left side), and a donor cell is being introduced into the oocyte using the injection needle (right side)

3.8 Cell Fusion and Activation of Enucleated Oocytes

6. Adjust the focus of microscope in the micromanipulator to get a good view of the oocytes.
7. Adjust the holding needle and injection needle to make them close to the oocytes.
8. Suck an oocyte using the holding needle. Turn the oocyte around using the injection needle to search for the polar body.
9. Position the polar body at the 5 o'clock position.
10. Place the injection needle at the 3 o'clock position of the oocyte and sucks out about an eighth of the surrounding cytoplasm (*see Note 4*, Fig. 2).
11. Suck one fibroblast cell into the injection needle and inject it into the oocyte at 3 o'clock.

1. Pre-warm fusion medium in a 38.5 °C water bath.
2. Add 2 mL of the pre-warmed fusion medium into each of three culture dishes (30 mm in diameter). Cover the fusion medium by applying 1.5 mL of paraffin oil.
3. Set the index of the BTX electro cell manipulator 2001 as follows: 2 DC pulses, 120 V and 30 µs pulse length.
4. Fill the fusion chamber with the fusion medium.
5. Transfer the reconstructed embryos (15 at a time) into a 30-mm culture dish containing 2 mL of the fusion medium to wash the embryos and wash the embryos for additional two times using two more 30-mm culture dishes containing 2 mL of fusion medium.
6. Pick up the reconstructed oocytes into the fusion chamber.



Align the oocytes to make an imaginary tangent line across the oocyte and donor cell parallel to the two electrodes.

Fig. 3 Align the oocytes to make an imaginary tangent line across the oocyte and donor cell parallel to the two electrodes in the chamber for electrical fusion

7. Position the reconstructed oocytes using a needle to make a straight line through the center of the oocyte and the fibroblast cell in the oocyte perpendicular to the electrode wire (Fig. 3).
8. Press the power button.
9. Pick up the reconstructed oocytes into a 30-mm culture dish containing 2 mL of the manipulation medium.
10. Wash the reconstructed embryos and incubate them in porcine zygote medium-3 with BSA (PZM-3 with BSA) at 38.5 °C in 5% CO₂ humidified air.
11. After 30 min incubation, discard the non-fused embryos and continue to culture the fused embryos at 38.5 °C in 5% CO₂-humidified air (50 reconstructed embryos in a 50-µL PZM-3 with BSA drop) (*see Note 5*).

3.9 Preparation of Embryo for Transfer

1. After about 18 h of culture, take the embryos from the incubator and examine under a microscope.
2. Pick up the live embryos and transfer them into a 30-mm culture dish containing 2 mL of PZM-3 with BSA (*see Note 6*).
3. Transfer the embryos into an embryo transport tube.
4. Load the transport tube into the embryo transport box and set the temperature of this box to 38.5 °C.
5. Transport the box to the pig farm for embryo transfer.

3.10 Embryo Transfer

1. Select naturally cycling sow of standing estrus on the day of nuclear transfer as the surrogate sow (*see Note 7*).
2. Anesthetize the surrogate sow by injecting propofol into the auricular vein of surrogate sow.
3. Maintain anesthesia with isoflurane using Anesthesia Machine for Large Animal.
4. Place the sow onto the operating table.
5. Tie the sow limes with ropes and keep abdomen upward.
6. Clean up the abdomen carefully.
7. Disinfect the area between the last and the penultimate pair of nipples evenly with tincture of iodine and 75% alcohol.
8. Make a 10-cm incision into the abdominal midline to expose the oviduct (Fig. 4).



Fig. 4 Embryo transfer into a surrogate sow by surgery

9. Transfer embryos from the embryo transfer tube into the manipulation medium and then load embryos into a Tomcat catheter attached to a 1 mL syringe.
10. Place the embryos in the ampullar region of oviduct by inserting 5 cm of the catheter through the ovarian fimbria and into the ampulla.
11. Suture the surgical wound carefully.
12. Apply penicillin (0.5 kU/kg) and streptomycin (1 kU/kg) on the wound and inject subcutaneously another dose of penicillin and streptomycin into the sow.
13. Check the pregnancy by ultrasonography on day 27 post SCNT and then monitor regularly at a 2-week interval.

4 Notes

1. Porcine fetal fibroblast cells could be obtained from fetuses of sow with 35–40 days of pregnancy [6]. Fetuses of sow with less days of pregnancy produce less amount of fibroblast cells. However, fibroblast cells generated from fetuses of sow with more days of pregnancy survive less passages although much more fibroblasts can be derived at the beginning. If the fibroblast cells need to be genetically modified, highly proliferative fibroblasts can be derived from fetuses at 30–34 days of gestation.
2. If COCs come from the ovaries of immature sows, their quality and maturation rate may decline. IGF1, LIF, and FGF2 have been showed to improve the maturation rate of COCs [33]. We recommend adding 15 ng/mL IGF1, 15 ng/mL LIF, and 25 ng/mL FGF2 into the oocyte maturation medium to improve the maturation of COCs from the ovaries of immature sows.
3. If the fetal fibroblast cells have been cultured for just a short period of time (<30 days) and passaged a few times (<7 passages) after derivation, it is recommended that the thawed cells be cultured before being used as nuclear donors for NT since the freshly cultured cells fuse at a higher rate. For cells cultured already for a long period of time, such as genetically modified cells, the attachment and proliferative ability becomes very low, thus the thawed cells should be used directly as donors for NT without further culture.
4. Since the condensed chromosomes are always located in the cytoplasm underneath the first polar body, enucleation of in vitro matured metaphase II oocytes can be performed by simply aspirating the first polar body and the adjacent

cytoplasm without staining the chromosomes. By using this “blind enucleation” method, the enucleation rate can reach up to between 85% and 90%.

5. Successful fusion of the reconstructed embryos is examined after 30 min incubation in CO₂ incubator after fusion. Discard the non-fused embryos. In order to harvest many more fused embryos, the non-fused embryos can be subject to a second round of fusion and activation.
6. A small portion of embryos would suffer death after culturing over a period of time. The dead embryos might affect the survival and subsequent development of the co-transferred live embryos. Therefore, embryos with swelling morphology, fragmented organelles or condensed cytoplasm should be discarded before embryo transfer.
7. To increase full-term development rate of the reconstructed embryos, selection of surrogate sows is also critical. For example, using the sows that have undergone more than one pregnancy as surrogates might help enhance pregnancy rate. Furthermore, the climate, physiological environment in the oviduct and/or uterus, and number of the transferred embryos could also affect full-term development of the reconstructed embryos.

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Chapter 2

Cloning of Monkeys by Somatic Cell Nuclear Transfer

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Abstract

Somatic cell nuclear transfer (SCNT) is a promising method to establish genetically modified monkeys with identical genetic background as models in biomedical research. We have recently cloned monkeys by optimization of the SCNT protocols and inclusion of the epigenetic modulator. Here, we describe the protocol for generation of cloned monkeys by somatic cell nuclear transfer.

Key words Monkey cloning, Somatic cell nuclear transfer, Genetically modified monkey models

1 Introduction

Owing to their high similarity in physiology and behavior, monkeys are widely used as a model to study human biology and diseases. With the development of gene-editing technologies, monkey models will play increasingly important roles in the future. Lentiviral cell transduction and nuclease-mediated gene-editing in early embryos are the two main methods to generate genetically modified monkey models. CRISPR/Cas represents the latest progress for the nuclease-mediated gene-editing in animal embryos. However, there are still several limitations for these two methods. For example, there are multiple and random integrations of transgenes in the lentivirus-based transgenic monkeys, and mosaicism in the CRISPR/Cas-edited monkeys, resulting in various genotypes for each individual founder monkeys. Another limitation is the inconsistency in genetic backgrounds among the transgenic monkeys, and this may lead to variations in experimental results among different monkeys. Cloning of macaque monkeys from a gene-edited somatic cell by somatic cell nuclear transfer (SCNT) allows the generation of genetically identical monkeys without mosaicism, and thus it has been considered as an ideal method to generate gene-modified monkey models.

Somatic cell nuclear transfer (SCNT), also known as cloning, can produce animals through transferring of a single somatic nucleus

into an enucleated oocyte, and has been intensively studied since John Gurdon successfully cloned frogs using nuclei from the intestinal epithelium cells of tadpoles [1]. After the successful cloning of Dolly the sheep in 1996 using SCNT [2], mammalian cloning has been achieved using similar methods in more than 20 different species including cows, mice, pigs [3–5], and others. The first cloning of cynomolgus monkeys was achieved by our group in 2017 using the fetal fibroblasts as nucleus donors [6]. Recently, we isolated the fibroblasts from the monkeys whose circadian transcription factor BMAL1 has been knocked out and used these fibroblasts as nucleus donors to generate five monkeys with both alleles harboring the *BMAL1* mutation without mosaicism [7].

This chapter provides the related protocols for cloning of monkeys using the key technology of somatic cell nuclear transfer. Since animal cloning relies on several techniques, we will describe the step-by-step procedures for the following integral experiments about monkey cloning: monkey superovulation (Subheading 3.1), oocyte collection (Subheading 3.2), preparation of monkey fibroblasts (Subheading 3.3), donor cell preparation (Subheading 3.4), somatic cell nuclear transfer (Subheading 3.5), monkey embryo transfer (Subheading 3.6), pregnancy test and monitoring (Subheading 3.7), as well as STR and SNP analyses (Subheading 3.8).

2 Materials

Prepare all the solutions using distilled water and analytical grade reagents.

2.1 Monkey Superovulation

1. Human recombinant follitropin.
2. Human chorionic gonadotrophin (hCG).

2.2 Oocyte Collection and Transfer

1. Zoletil (Virbac).
2. Iodine tincture.
3. 75% medical ethanol.
4. HEPES-buffered Tyrode's Albumin Lactate Pyruvate (TALP) medium: 6.660 g/L NaCl, 0.239 g/L KCl, 0.294 g/L CaCl₂·2H₂O, 0.102 g/L MgCl₂·6H₂O, 0.048 g/L Na₂HPO₄, 0.900 g/L Glucose, 1.32 g/L Sodium lactate, 0.010 g/L phenol red, 0.168 g/L NaHCO₃, penicillin-G (sodium salt)/streptomycin (100×, 10 mL), 2.603 g/L HEPES, and 0.060 g/L sodium pyruvate. Adjust the pH to 7.4.
5. TH3 medium: HEPES-buffered TALP medium, containing 0.3% bovine serum albumin (FBS) [8].
6. 2.5% Heparin: 1 mg heparin dissolved in 39 mL TH3 medium.

7. Complete CMRL-1066 medium: CMRL Medium 1066 supplemented with 10% FBS, 40 mg/L sodium pyruvate, 150 mg/L glutamine, 550 mg/L calcium lactate, and 400 µg/L estradiol.
8. Tube heater (Cook).
9. Oscillation Mixer (Kylin-Bell Lab Instruments, QH-600).
10. Shadow-less lamp.
11. Operation table.
12. Laparoscope operation system:
 - (a) Laparoscope (Olympus, OVT-SC2).
 - (b) Light source (Olympus, CLH-250).
 - (c) Monitor.
 - (d) Vacuum aspiration system (Cook).
 - (e) Veress needle (Olympus).
 - (f) Trocar (Olympus).
 - (g) Grasping forceps (Olympus).
13. Electric stimulator.

2.3 Fibroblast

Culture and Donor Cell Preparation

1. 0.25% Trypsin-EDTA.
2. Dimethylsulfoxide (DMSO).
3. Dulbecco's Modified Eagle Medium (DMEM).
4. Fibroblast culture medium: DMEM containing 100 IU/mL penicillin and 100 µg/mL streptomycin, 15% FBS, MEM non-essential amino acids (0.1 mM glycine, 0.1 mM L-alanine, 0.1 mM L-asparagine, 0.1 mM L-aspartic acid, 0.1 mM L-glutamic acid, 0.1 mM L-proline, and 0.1 mM L-serine), 2 mM glutamax, and 1 mM sodium pyruvate.
5. Fibroblast freezing medium: 90% FBS with 10% DMSO.
6. Benchtop centrifuge.

2.4 Somatic Cell Nuclear Transfer

1. Manipulation solution: TH3 containing 5 µg/mL cytochalasin B.
2. Hamster embryo culture medium-9 (HECM-9): 113.6 mM NaCl, 3 mM KCl, 1.9 mM CaCl₂·2H₂O, 0.5 mM MgCl₂·6H₂O, 25 mM NaHCO₃, 4.5 mM sodium lactate (60% syrup). Add 6.639 g NaCl, 0.224 g KCl, 0.279 g CaCl₂·2H₂O, 0.102 g MgCl₂·6H₂O, 0.632 mL sodium lactate (60% syrup), 0.1 g polyvinyl alcohol (PVA), and 2.1 g NaHCO₃ to 1 L distilled water, and adjust the pH to 7.4 [9].
3. 100× Amino acid-pantothenate stock: 50 mM taurine, 1 mM asparagine, 1 mM cysteine, 1 mM histidine, 1 mM lysine, 1 mM proline, 1 mM serine, 1 mM aspartic acid, 1 mM glycine, 1 mM glutamic acid, 20 mM glutamine, and 0.3 mM pantothenate.

4. HECM-9aa medium: Add amino acid–pantothenate stock to HECM-9 medium at a ratio of 1:100 before use.
5. HECM-9aa with 5% FBS: used for the culture of SCNT embryos after the 8-cell stage.
6. 10% PVP: dissolve 1 g polyvinyl pyrrolidone (PVP) into 10 mL TH3 medium.
7. 100× cytochalasin B stock: 5 mg/mL cytochalasin B dissolved in DMSO.
8. 100× Trichostatin A (TSA) stock: 10 µM TSA dissolved in DMSO.
9. Fusogenic viral envelop (HVJ-E) (COSMO BIO CO. LTD.).
10. Ionomycin.
11. 6-Dimethylaminopurine.
12. Activation medium: HECM-9aa medium containing 2 mM 6-dimethylaminopurine and 10 µM TSA.
13. Mineral oil.
14. Cell strainers with 40 µm mesh size.
15. Capillary glass (Sutter Instrument, B100-75-10).
16. Micropipette puller (Sutter Instrument, P97).
17. Microgrinder (Narishige, EG-400).
18. Microforge (Narishige, MF-900).
19. Insufflator.
20. Stereomicroscope.
21. Inverted microscope.
22. Laser system (Hamilton Thorne).
23. Piezo impact drive system (Prime Tech).
24. Spindle imaging microscopic system (Oosight).
25. Micromanipulators (Narishige).
26. 1 mL Syringe.
27. Microinjector (Narishige, IM300).
28. Microscope heating stages (Tokai Hit).

2.5 Pregnancy Test and Monitoring

1. Ultrasound instrument.
2. Restraint chair.
3. Electric stimulator.

2.6 STR and SNP Analysis

1. Genomic DNA extraction kit.
2. PCR thermal cycler.
3. mtDNA primers for SNP analysis:

- (a) mtDNA forward: CCACATTCACATCAAACCATCACTT.
- (b) mtDNA reverse: CAAGCAGCGAATACCAGCAAAA.

3 Methods

3.1 Monkey Superovulation

1. Monitor and document the menstruation cycles of the female monkeys and select those with normal menstruation cycles as candidates.
2. From the third day of the menstrual bleeding, intramuscularly inject 18.75–37.5 IU (depending on the weight and ovarian response of the selected monkeys) of recombinant human follitropin twice a day for 7–8 days.
3. Examine the ovarian response after superovulation on the tenth day of the menstrual bleeding through ultrasonography. If the ovary diameter is smaller than 5 mm with no obvious follicles, abandon the superovulation process.
4. Inject 1000 IU of human chorionic gonadotrophin (hCG) intramuscularly to the monkeys on the 11th day of menstrual bleeding. Inject 1500 IU of hCG if the monkey is heavier than 5 kg.

3.2 Oocyte Collection

1. Prepare complete CMRL-1066 medium as described in Sub-heading 2 the day before oocyte collection and equilibrate the medium overnight in an incubator set at 37 °C and 5% CO₂. Anesthetize the superovulated monkey by intramuscularly injecting 5–10 mg/kg Zoletil 35–37 h after hCG injection (Fig. 1a).
2. Shave the fur in the abdomen and clean the shaved skin first with iodine tincture and then with 75% medical ethanol.
3. Constrain the monkey on the operation table by binding their arms and legs to the table legs.
4. Cover the monkey with a piece of aseptic hole-towel with the abdomen exposed.
5. Insert a Veress needle into the abdominal cavity through the skin above the navel. Inject N₂ to inflate the abdominal cavity at a rate of 1 L/min to 15 mmHg pressure and stabilize it. Replace the Veress needle with trocar into abdominal cavity and remove obturator, leaving the cannula connected with the gas tube.
6. Insert the laparoscope through the cannula, adjust its position to find the ovaries. Inserting two accessory ports from the left and right side of the lumbar region according to the position of ovaries, respectively.

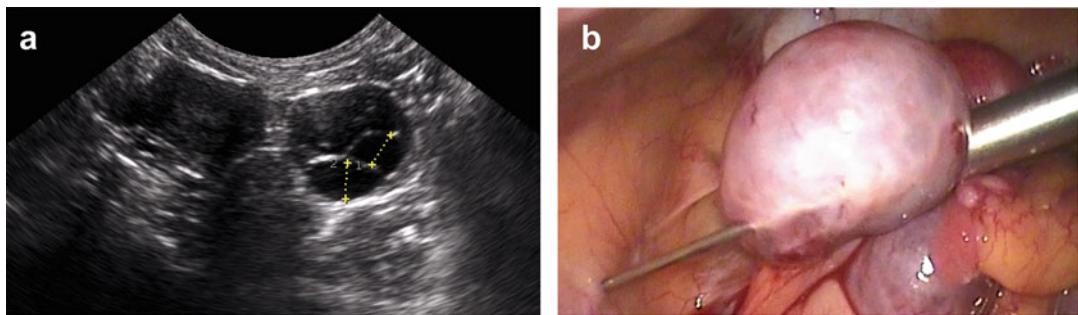


Fig. 1 Monkey superovulation and oocyte collection. (a) The ultrasonography of the follicles on day 11 of menstrual bleeding before injection of hCG. (b) Surgical aspiration of follicle fluid

7. Insert a trocar into the abdominal cavity at one side. Pull out obturator and insert the grasping forceps into abdominal cavity. Grasp the ovary and adjust it to a proper position for the subsequent oocytes collection.
8. Fill a 15-mL centrifuge tube with 2–3 mL of TH3 supplemented with 2.5% heparin and pre-warm it using the 37 °C tube heater. Put the Teflon-tubing end of the 18-G follicle aspiration needle into the 15-mL centrifuge tube prepared above. Insert the needle end of the follicle aspiration needle system into the abdominal cavity. To prevent the blood from coagulation, load the 15-mL follicle-collecting centrifuge tube onto an oscillation mixer and keep the mixer running during the whole process after the aspiration process starts. After positioning the needle to the follicles via the imaging system, stab and aspirate all follicles within the two ovaries (Fig. 1b).
9. After follicle aspiration, sew up the muscle layer and skin of incisions separately with absorbable and silk suture, respectively. Sterilize the incision with iodine tincture.
10. Add 1 mL hyaluronidase (2 mg/mL dissolved in TH3 medium) into the follicular aspirates and gently pipette the mixture to promote digestion of the cumulus cells. Filter the follicular aspirates using a cell strainer with a mesh size of 40 µm to remove the blood and cumulus cells. The oocytes are left on the strainer. Wash the follicles in the cell strainer with 3-5 mL TH3 medium in order to remove further the hyaluronidase.
11. Turn over the cell strainer into a clean 6-cm dish and wash off the oocytes with the TH3 medium. Collect all the medium in the 6-cm dish.
12. Pick up the oocytes under the stereomicroscope using an oral suction glass pipette with an orifice diameter of ~300 µm. Culture the collected oocytes in the complete CMRL-1066 medium to promote their maturation before enucleation (*see Note 1*).

3.3 Monkey Fibroblasts Preparation

Monkey fibroblasts can be derived from both the aborted fetus of cynomolgus monkey (**steps 1–3**) or postnatal cynomolgus monkeys (**steps 4–6**).

1. For the monkey fetal fibroblast cells, peel all the skin of the fetus and cut them into small pieces.
2. Put the tissues into cell culture medium with DNase (1 mg/mL) and collagenase IV (0.5 mg/mL) and digest the tissues at 37 °C in 5% CO₂ for 4 h. Add cell culture medium to terminate the digestion and pipette the cell suspension to obtain single-cell fibroblasts. Filter the cell suspension with a cell strainer to exclude indigested cell mass. Plate the fibroblasts at a proper density into a 10-cm dish and record the passage number as P0.
3. The next day examine the culture and make sure the fibroblasts attach to the dish. Refresh the cell culture medium daily until the cell cover 80–90% area of the dish, and then perform cryopreservation (**step 7**).
4. For the postnatal monkeys, cut a small piece of skin from the anesthetized monkey. Wash the tissue with phosphate-buffered saline containing penicillin and streptomycin for three times before cutting them into small pieces (1–2 mm³).
5. Put these tissues into a 6-cm culture dish and add a proper volume of cell culture medium so that all of the skin tissues will be covered by the buffer and no minced skin tissues will float in the buffer. Usually, the fibroblasts will migrate away from the tissues after 7 days of culture (Fig. 2a, *see Note 2*).
6. When fibroblasts cover 80% area of the dish, digest the fibroblasts with 0.25% trypsin-EDTA for 1 min. After washing the cells by centrifugation, passage fibroblasts into a 10-cm dish in the fibroblast culture medium.
7. Culture the isolated fibroblasts in the 10-cm culture dish until they cover 80–90% area of the dish. Add 1 mL of 0.25% trypsin-EDTA, incubate at 37 °C for 1 min to detach the fibroblasts, and then add 2 mL cell culture medium to terminate digestion.
8. Centrifuge the cell suspension for 3 min at 200 × g and discard the supernatant. Resuspend the cell pellet with the precooled cell freezing medium containing 10% DMSO and 90% FBS.
9. Put the cells into a freezing container and then place the container inside a –80 °C freezer to initiate freezing. The next day transfer the cells into liquid nitrogen for long-term storage until use.

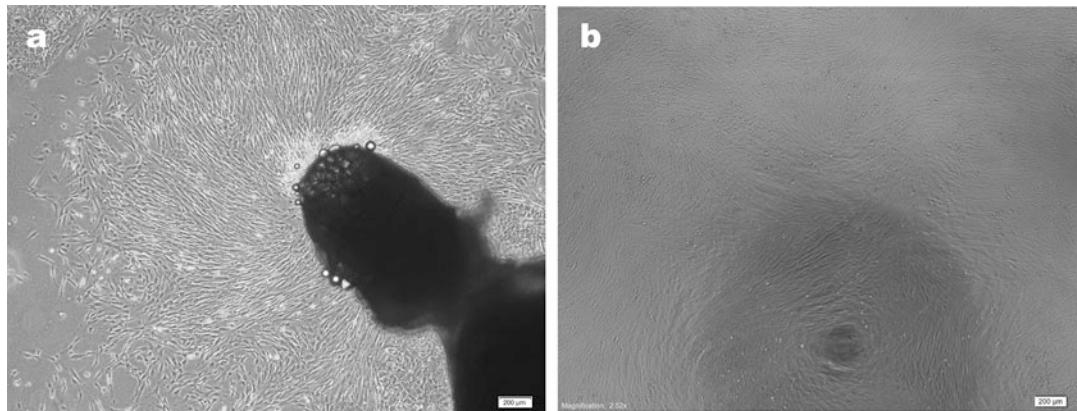


Fig. 2 Preparation of monkey fibroblasts. **(a)** The fibroblasts emigrate from the monkey skin tissue. **(b)** The fibroblasts at confluence before SCNT

3.4 Preparation of Donor Cells

1. Take one vial of fibroblasts from liquid nitrogen and quickly thaw the cells in a 37 °C water bath. Transfer the cells into a 15-mL centrifuge tube immediately. Add 3–5 mL of cell culture medium into the tube and mix gently.
2. Centrifuge the cell suspension for 3 min at $200 \times g$ and then discard the supernatant. Suspend the cell pellet with cell culture medium and passage them onto 2–4 wells of a 48-well plate with the different cell densities as follows: 5×10^4 , 3×10^4 , 2×10^4 , and 1×10^4 cells/well (*see Note 3*).
3. Culture the thawed cells at 37 °C in 5% CO₂ until confluence to arrest the cells at G0 phase (Fig. 2b). Before use, digest the fibroblasts with 0.25% trypsin-EDTA and incubate at 37 °C for 1 min. Then, add two-fold volume of the fibroblast culture medium to terminate the trypsin digestion.
4. Centrifuge the cell suspension for 3 min at $200 \times g$ and discard the supernatant. Pipet the cell pellet with PBS to wash for three times, centrifuge the cell suspension for 3 min at $200 \times g$ each time, and then discard the supernatant each time. Mix the remaining pellet through pipetting, which is about 20 µL. Use the cell suspension for the following nuclear injection.

3.5 Somatic Cell Nuclear Transfer

The SCNT procedure, which can be divided into enucleation of oocyte, fusion with somatic cell, and mRNA injection, is performed by using an inverted microscope equipped with piezo impact drive system and laser system. The left micromanipulator controls the micropipette holder that is connected with a holding pipette. The right micromanipulator controls the micropipette holder that is connected with either a blunt-tip enucleation pipette or an injection pipette. The holding pipette and enucleation/injection pipette are controlled by IM9B and CellTram vario syringes with filled oil, respectively.

1. Prepare the enucleation pipette, injection pipette, and RNA injection pipette using capillary glass and P97 micropipette puller. All the above pipettes are pulled by thin-wall borosilicate tubing without filaments (B100-75-10). The enucleation pipette is processed to 8–10 μm outer and 7–9 μm inner diameters using MF-900 microforge. Load the Mercury (~2 mm) into the back end of the enucleation pipette through a 1 mL syringe. The injection pipette used for injecting the fibroblasts is processed to 16–18 μm outer and 15–17 μm inner diameters. Load the injection pipette on the EG-400 microgrinder at 45° angle and grind the tip to make a 45° tilt. The mRNA injection pipettes are processed to 4–5 μm outer and 3 μm inner diameters with Mercury (~2 mm) loaded.
2. Prepare a glass-bottom dish containing a few drops of 10–15 μL manipulation solution and a drop of 3 μL PVP solution. Cover all drops of solutions with mineral oil. Set the holding pipette and the enucleation pipette to a proper position and wash the enucleation pipette in the PVP with a relatively high speed (>5) and power (>5) controlled by piezo unit (*see Note 4*).
3. Transfer around 10 MII oocytes to one manipulation drops; use spindle imaging microscopic system (Oosight) to observe the spindle of oocytes. The spindle looks like a small bright sheet under the Oosight (Fig. 3a).
4. Hold the oocyte using the holding pipette to a proper position and adjust the spindle and align the enucleation pipette to the same horizontal plane. Penetrate the zona pellucida using the piezo unit with a moderate speed (2–3) and power (2–3). Suck the spindle into the enucleation pipette with as little cytoplasm as possible.
5. Add 2.5 μL fusogenic viral envelop proteins (HVJ-E) to one manipulation drop. Incubate the donor cells in this manipulation drop for a few seconds. Transfer the enucleated oocytes into the manipulation drop and generate a slit in the zona pellucida using laser irradiation. Use the injection pipette to introduce one donor cell into the perivitelline space of one enucleated oocyte and transfer all of the reconstructed embryos back to the pre-equilibrated HECM-9aa at 37 °C under 5% CO₂ (Fig. 3d, e, *see Note 5*).
6. After 1–2 h of cell fusion, transfer the reconstructed embryos into the TH3 medium containing 5 mM ionomycin and incubate for 5 min; and then transfer the embryos into the activation solution and incubate for 5 h to activate the oocytes. After activation, the SCNT embryos are transferred into the HECM-9aa medium containing 10 μM TSA and incubate for 3–5 h. After the TSA treatment, transfer the embryos back into the HECM-9aa medium.

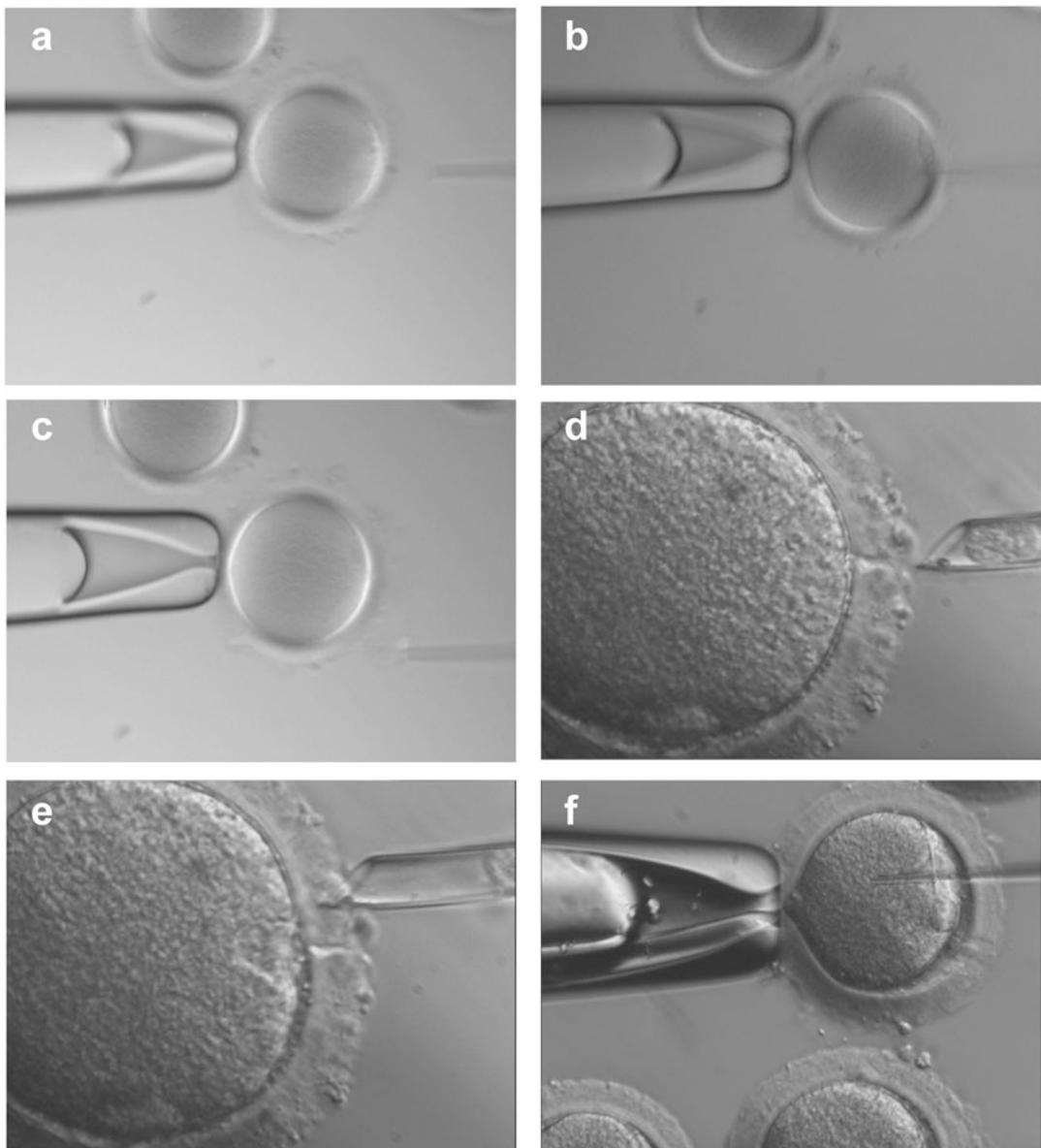


Fig. 3 The SCNT procedure. (a–c) The enucleation process. The spindle and chromosome complex of MII oocyte is removed by enucleation pipette under microscope with an Oosight spindle view. (d, e) The donor injection and fusion. Fibroblast is injected into the perivitelline space of the enucleated oocyte. (f) The Kdm4d mRNA is injected to the cytoplasm of the reconstructed SCNT embryos

7. Six hours after activation, load the mRNA injection pipette on the right manipulator connected pipette holder and wash the pipette in the PVP drop. Transfer the activated reconstructed zygotes into the manipulation drop and inject 10 pL Kdm4d mRNA with the concentration above 1000 ng/ μ L (Fig. 3f). Culture the SCNT embryos in the HECM-9aa medium at

37 °C in 5% CO₂. The embryos are transferred to the HECM-9aa medium containing 5% fetal bovine serum (FBS) after reaching the eight-cell stage, change the medium every other day until the embryos reach to the blastocyst stage.

3.6 Monkey Embryos Transfer

The embryos are transferred during the two-cell stage to the blastocyst stage.

1. Use adult female monkeys with synchronous menstruation cycles as surrogate candidates (*see Note 6*).
2. Conduct anesthetization and laparoscopic surgery as described in oocyte collection (Subheading 3.2, steps 1–6). Examine the ovaries of surrogate candidates using endoscope. Select those with mature follicle, stigma, or fresh corpus luteum as surrogates.
3. Insert trocar into abdominal via paralumbar fossa near the ovary. Expand the incision to 1–2 cm with a sterile scissor. Seize the adipose tissues that associate with ovary with a clamp and pull out the ovary under the shadow-less lamp.
4. Transfer 4–7 reconstructed embryos into an oral suction glass pipette. Insert the tip of the oral suction glass pipette into the oviduct through the fimbriae, and blow the embryos into the oviduct. Check the tip of the glass pipette under stereomicroscope to make sure that all the embryos have been transferred into the oviduct.
5. Sew up the incision as described in the section for oocyte collection (Subheading 3.2, step 9).

3.7 Pregnancy Test and Monitoring

Conduct ultrasonic examination on the surrogate mothers after 25 days of embryo transfer to examine the pregnancy status. At that time, fetal heart beats can also be detected. Monitor the pregnancies after 130 days. If there are twin fetuses or other abnormal situations such as fetal malposition, cesarean surgery will be needed.

3.8 STR and SNP Analysis

1. Take the ear tip tissue of the oocyte donor monkeys, somatic cell donor monkeys, surrogate monkeys, and the cloned monkeys. Extract genome DNA from each sample.
2. For short tandem repeats (STR) analysis, the locus-specific primers with fluorescent label (FAM/HEX/TMR) were designed for the PCR amplification. The amplicons are subsequently diluted and mixed with the internal size standard ROX500, then load them on the ABI PRISM 3730 genetic analyzer for capillary electrophoresis. Analyze the raw data with the program Gene Marker 2.2.0, which produces wave plots, Excel documents, and DNA profiles.

3. For single nucleotide polymorphism (SNP) analysis, a pair of primers named mtDNA forward and mtDNA reverse are designed to amplify mtDNA through PCR. The PCR products are subjected to sequencing subsequently followed by the SNP analysis.

4 Notes

1. The monkey oocytes we collected are in different developmental stages. Usually, we can obtain MII, MI, or GV oocytes. The CMRL-1066 medium can promote development of some MI and GV oocytes into MII stage. Thus, all the oocytes are cultured in the complete CMRL-1066 medium before enucleation.
2. For this primary culture step, monitor the tissue and prevent them from floating. Usually, most of the minced tissues will attach to the bottom of the dish in the second day. Then, add some cell culture medium in the second or third day. Refresh the cell culture medium every 2–3 days before the next step.
3. The same preparation of fibroblasts can be used for several days once thawed. Plate the cells at various densities so that the cells will reach confluence at different time.
4. Microscopes are equipped with a thermo plate. Make sure the temperature of the thermo plate is maintained at 37 °C during the whole experiment process.
5. Usually, we select the smaller fibroblasts as donor cells since they are more likely to be in the G0 phase.
6. Usually, monkeys of day 13 to day 18 post ovulation can be chosen as proper surrogate candidates. The monkeys of day 14 to day 16 post ovulation are better hosts based on our experience.

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Chapter 3

Production of Cardiomyocyte-Like Cells by Fibroblast Reprogramming with Defined Factors

Emre Bektik and Ji-Dong Fu

Abstract

Over the last decade, great achievements have been made in the field of direct epigenetic reprogramming, which converts one type of adult somatic cells into another type of differentiated cells, such as direct reprogramming of fibroblasts into cardiomyocytes, without passage through an undifferentiated pluripotent stage. Discovery of direct cardiac reprogramming offers a promising therapeutic strategy to prevent/attenuate cardiac fibrotic remodeling in a diseased heart. Furthermore, in vitro reprogramming of fibroblasts into cardiomyocyte-like cells provides new avenues to conduct basic mechanistic studies, to test drugs, and to model cardiac diseases in a dish. Here, we describe a detailed step-by-step protocol for in vitro production of induced cardiomyocyte-like cells (iCMs) from fibroblasts. The related procedures include high-quality fibroblast isolation of different origins (neonatal cardiac, tail-tip, and adult cardiac fibroblasts), retroviral preparation of reprogramming factors, and iCM generation by fibroblast reprogramming via retroviral transduction of Gata4, Mef2c, and Tbx5. A detailed written protocol will help many other laboratories, inexperienced in this area, to use and further improve this technology in their studies of cardiac regenerative medicine.

Key words Cardiomyocytes, Fibroblasts, Epigenetic reprogramming, Transdifferentiation, Transcription factors, Heart regeneration, Cell therapy

1 Introduction

Cardiovascular diseases remain the leading cause of death worldwide [1]. Coronary artery disease is the most common form of cardiovascular diseases, resulting in the loss of cardiomyocytes (CMs) at the site of ischemic injury. To compensate for the loss of CMs, cardiac fibroblasts quickly respond to injury and initiate cardiac remodeling in an injured heart [2–4], which leads to dysfunction of the heart and eventually a heart failure. Heart transplantation remains the final solution for patients with an end-stage heart failure, but is limited by the shortage of donor organs. Cellular therapy offers more accessible options for a broader group of coronary heart patients and prevents a diseased heart from end-stage failure, and has been investigated with different

strategies, including transplantation of autologous adult stem cells [5, 6] or CMs derived from embryonic stem cell (ESC) or induced pluripotent stem cell (iPSC) [7, 8], activation of endogenous progenitors [9–11], cell cycle reentry of adult CMs [12–14], and direct epigenetic reprogramming [15–17].

Since lost CMs in an injured heart are replaced by cardiac fibroblasts, it will be a promising therapy for cardiac regenerative medicine if those proliferated cardiac fibroblasts can be transdifferentiated into functional CMs. Transdifferentiation was initially reported in the early 1990s that MyoD alone converts fibroblasts and epithelial cells into skeletal muscle cells [18]. After decades of efforts, the discovery of iPSCs [19] demonstrated that, rather than a single transcription factor, a combination of several defined transcription factors might be required to directly convert a type of terminally differentiated somatic cells into another type of cells. Indeed, in 2010, Ieda et al. [15] successfully identified a combination of three transcription factors (GMT: *Gata4*, *Mef2c*, and *Tbx5*) that can convert cardiac fibroblasts directly into induced cardiomyocyte-like cells (iCMs) without going through an intermediate pluripotency or progenitor state. Soon after the first discovery, in vitro mouse cardiac reprogramming by different combinations of defined factors [17, 20–22], in vivo mouse iCM-reprogramming [16, 17, 23], and in vitro human iCM-reprogramming [24–27] had been achieved in different laboratories around the world. Since then, many efforts have been invested to improve the efficiency and efficacy of reprogramming by manipulations of signaling pathways [28–30] epigenetic barriers [31], cell cycle regulation [32, 33], chemokine signaling [34], and inflammatory immune signaling [35]. Those progresses have been reviewed in recent publications [36, 37].

Despite the success of multiple groups, it remains challenging to achieve a high efficiency of in vitro reprogramming, which requires high quality of cultured fibroblasts and robust expression of all reprogramming factors in individual fibroblasts [38]. In vitro iCM-reprogramming has significant advantages to study mechanisms of epigenetic reprogramming, to test chemicals for drug development, and to model cardiac diseases in a dish in the future; therefore, the detailed written protocol is important to help many research laboratories master it as well as to inspire further refinement of this technology.

This chapter provides step-by-step protocols for: (1) isolation and culture of high-quality starting cells for reprogramming (i.e., neonatal cardiac, neonatal tail-tip, and adult cardiac fibroblasts); (2) preparation of high-titer retroviruses encoding the reprogramming factors; and (3) generation of iCMs from fibroblasts transduced with retroviruses encoding GMT.

2 Materials

1. α MHC-GFP transgenic mice (Gladstone Institutes, Dr. Deepak Srivastava laboratory) (*see Note 1*).
2. Anti-Mouse CD90.1 (Thy-1.1) APC.
3. Blasticidin.
4. Fetal bovine serum (FBS) (Hyclone, Cat.# SH30910).
5. FuGENE® 6 Transfection Reagent.
6. FACS buffer: 2% FBS in PBS with 2 mM EDTA, store at 4 °C.
7. Fibroblast Explant Medium: combine 395 mL of Iscove's Modified Dulbecco's Medium (IMDM), 100 mL FBS, and 5 mL Penicillin/Streptomycin. Store at 4 °C. Warm up before use.
8. Gelatin 0.1% (wt/vol) solution.
9. iCM-Reprogramming Medium: combine 355 mL DMEM, 85 mL Medium 199 (M199), 50 mL FBS, 5 mL non-essential amino acids, and 5 mL Penicillin/Streptomycin. Store at 4 °C. Warm up before use.
10. Nalgene syringe filter, 0.45- μ m pore-size, SFCA-membrane.
11. Opti-MEM I reduced-serum medium.
12. PBS without Ca²⁺ and Mg²⁺.
13. Plat-E Medium: combine 445 mL Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine, 5 mL non-essential amino acid solution (NEAA, 100×), and 50 mL FBS. Store at 4 °C. Warm up before use.
14. Hexadimethrine bromide (Polybrene).
15. Propidium iodide.
16. Puromycin.
17. 0.05% (wt/vol) Trypsin/EDTA.
18. Plasmids: pMX-Gata4, -Mef2c, -Tbx5, and -dsRed (Gladstone Institutes, Dr. Deepak Srivastava laboratory).

3 Methods

To achieve a high efficiency of iCM-reprogramming, it requires freshly purified fibroblasts with high-quality and fresh retroviruses with high titers; therefore, all experiments should be coordinated and started as planned (Fig. 1).

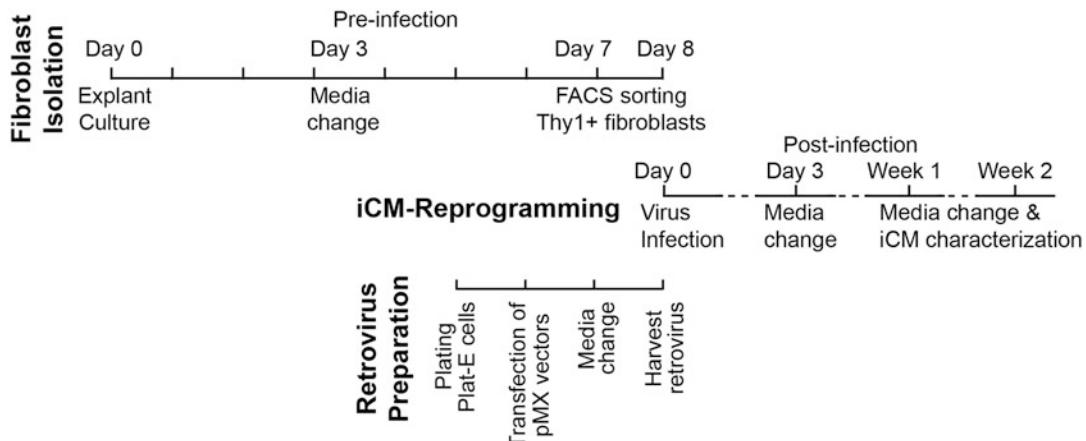


Fig. 1 Scheme of producing cardiomyocytes by epigenetic reprogramming of fibroblasts, including fibroblast isolation, retrovirus preparation, and reprogramming of induced cardiomyocytes (iCMs)

3.1 Fibroblast Isolation by Explant-Culture Method

Contamination could occur in primary cell culture from the freshly harvested tissues; therefore, all surgical tools (e.g., scissors and forceps) must be sterilized before experiments. Cell isolation should be carried out in a sterile biosafety level II tissue culture hood.

1. Coat cell culture dishes with 0.1% gelatin solution at 37 °C for >1 h (*see Note 2*).
2. Dissect hearts and/or collect tail-tips from neonatal pups (between postnatal 0.5–1.5 days) or adults (age: >8 weeks) of the αMHC-GFP transgenic mice. Put the harvested hearts/tail-tips in cold PBS and keep them on ice before fibroblast isolation (*see Note 3*).
3. Use a fluorescent dissecting microscope to examine for GFP expression in the hearts and identify the αMHC-GFP⁺ animals. After identification of the αMHC-GFP⁺ animals, use the tissues of hearts or tail-tips from the αMHC-GFP⁺ mice for fibroblast isolation (*see Note 4*).
4. Right before cell isolation, use a curved forceps to transfer tissues (i.e., hearts and/or tail-tips) of the αMHC-GFP⁺ mice into a dish that contains 70% ethanol. After 3–5 s in the 70% ethanol, quickly transfer the tissues into another dish containing cold PBS to wash out the residual ethanol, and then transfer the tissues into a third dish containing cold PBS. These wash steps could reduce the chance of bacterial contamination of the cell culture.
5. Transfer the hearts or tail-tips into a 35-mm dish using a curved forceps, and mince tissues into small pieces with a curved scissors (*see Note 5*).

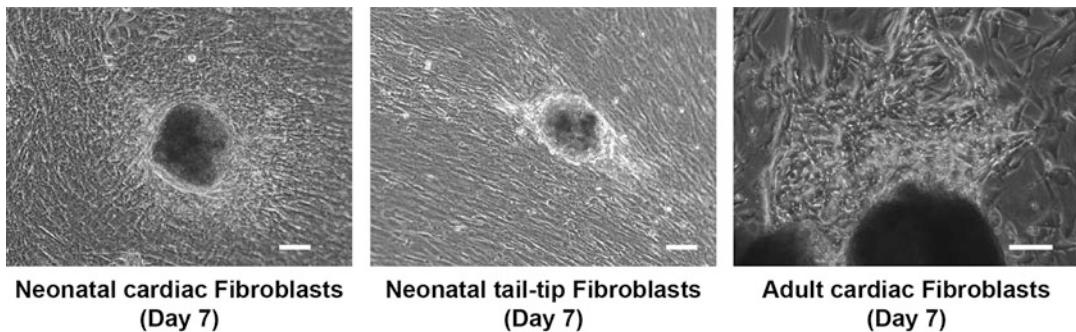


Fig. 2 Representative pictures of cultured neonatal cardiac fibroblasts and tail-tip fibroblasts and adult cardiac fibroblasts after 7 days of explant culture. Bars indicate 50 μm

6. Add proper amount of the fibroblast explant medium to the minced tissues (*see Note 6*) and then dissociate the tissue pieces by pipetting up and down gently.
7. Aspirate the coating gelatin solution from the dishes and then plate the suspended minced tissues evenly on the coated dishes (*see Note 7*).
8. Carefully transfer the dishes into a cell culture incubator (37 °C, 5% CO₂) and culture for 2–4 h to allow the minced tissue to settle (*see Note 8*).
9. Carefully take out the dishes from the incubator. Put the pipette onto the wall of the dish and add 9 mL of the fresh fibroblast explant medium VERY GENTLY into a 100-mm dish, or 3 mL media for a 60-mm dish (*see Note 9*).
10. Carefully and slowly transfer dishes into the incubator and avoid any turbulence. Culture the tissues for 3 days WITHOUT any disturbance.
11. Afterward, take out the dishes and move dishes back and forth gently to suspend the unattached tissues (*see Note 10*). Aspirate media along with the unattached tissues. Attach the pipette on the wall of the dish and gently add fresh fibroblast explant medium (10 mL per 100-mm dish or 3 mL per 60-mm dish).
12. Culture for 3–4 days to allow more fibroblasts to migrate out from the attached tissues (*see Note 11*). Figure 2 shows representative images of the fibroblasts from the neonatal hearts, neonatal tail-tip tissues, and adult hearts 7 days after explantation.

3.2 Fibroblast Purification by Fluorescence-Activated Cell Sorting (FACS)

The health and freshness of the fibroblasts are critical to achieve a high efficiency of iCM-reprogramming; therefore, explant-cultured fibroblasts are generally purified by FACS at Day 7 and could also be performed at Day 6 if there are sufficient fibroblasts from the

cultured tissues. Fresh fibroblasts from the explanted tissue dishes (P0 fibroblasts) should be used for reprogramming (*see Note 12*).

1. Aspirate the culture media and add 5 mL PBS into one 100-mm dish of the cultured cells to wash out the residual culture media. Aspirate PBS and repeat the PBS wash twice.
2. Aspirate PBS and add 2 mL trypsin (0.05%) into one 100-mm dish. Digest for 5 min at 37 °C. Take out the dishes from the incubator and gently pat the wall of the dishes. Examine the cells under a microscope to check if over 80% of the cells become round in shape and are detached from or loosely attached to the dishes.
3. Quench the digestion with 8 mL of fibroblast explant medium and pipette up and down to break up clumps into single cells.
4. Harvest the digested cells and pass the cells through a 40-µm cell strainer to remove undigested cell clumps and collect the filtered single cells into a 15-mL tube. Centrifuge the fibroblasts for 3 min at $300 \times g$.
5. Aspirate off the media and resuspend the cell pellet in 5 mL PBS. Centrifuge the cells for 3 min at $300 \times g$.
6. Resuspend the cell pellet in 1 mL FACS buffer (*see Note 13*). Take an aliquot of the resuspended cells (25 or 50 µL). Add this aliquot into 0.5 mL FACS solution and keep it on ice without staining of Thy1, which will be used as the negative control in FACS assay.
7. Add 20 µL anti-mouse Thy1-APC (1:50 dilution) in the resuspended cells and mix gently by finger tapping. Protect the tube from light and incubate for 30 min at room temperature.
8. Add 9 mL PBS and centrifuge the cells for 3 min at $300 \times g$. Repeat the PBS wash twice.
9. Resuspend the cells in 1 mL FACS buffer and add propidium iodide (50 µg/mL) into the Thy1-stained fibroblasts and the aliquoted unstained cells. Keep the stained cells on ice and protect them from light until FACS sorting. In general, more than 70% of the cells should be stained as Thy1⁺/αMHC-GFP⁻ fibroblasts (Fig. 3), which will be sorted and collected into a tube with 0.5 mL of the fibroblast explant medium.
10. Centrifuge the purified Thy1⁺/αMHC-GFP⁻ fibroblasts for 3 min at $300 \times g$ and then resuspend the cells with the fibroblast explant media; plate the fibroblasts in a gelatin-coated 6-well plate at a density of $10^4/\text{cm}^2$, at around $1-1.2 \times 10^5/\text{well}$ of a 6-well plate in 2 mL media. Those sorted fibroblasts will be used for iCM-reprogramming the next day by retroviral transduction of GMT.

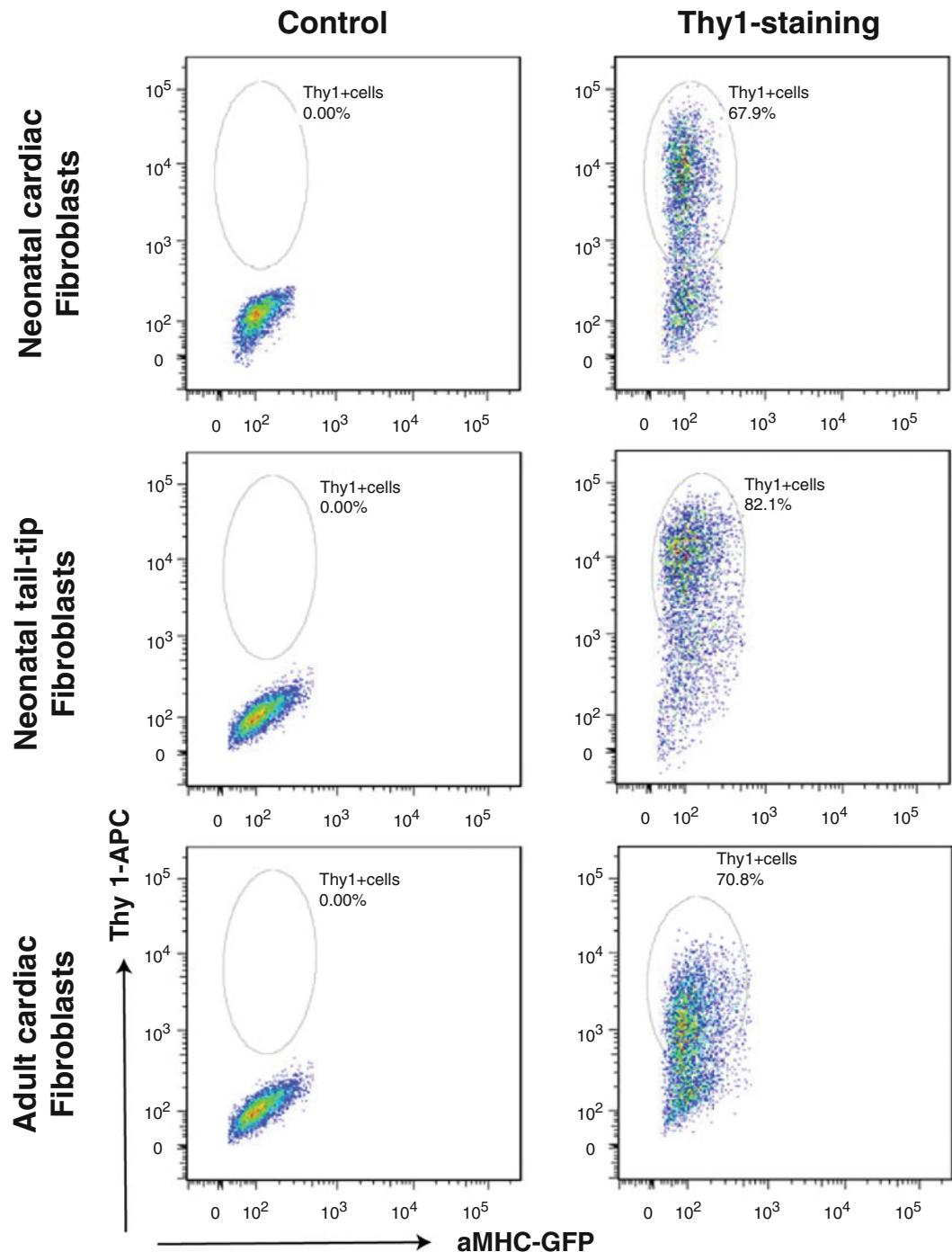


Fig. 3 Representative plots of FACS assays after Thy1-APC staining to purify fibroblasts

3.3 Retrovirus Preparation

The platinum-E (Plat-E) cells, a derivative of the HEK293T cell line, was established by using the packaging constructs with the EF1 α promoter, which ensures stable and robust expression of the retroviral structure proteins (gag, pol, and ecotropic env). In order to maintain robust expression of those retroviral structure proteins, the Plat-E cells are cultured with the Plat-E cell medium in the presence of puromycin (1 μ g/mL) and blasticidin (10 μ g/mL), which will selectively kill cells that have lost the expression of the packaging constructs. The Plat-E cell line is designed for rapid and transient production of high-titer ecotropic retroviruses (*see Note 14*).

1. Aspirate culture media and add 5 mL PBS into the cultured Plat-E cells to wash out the residual culture media; aspirate PBS and repeat the PBS wash once again.
2. Aspirate PBS and add 2 mL trypsin (0.05%) into the 100-mm dish; digest the Plat-E cells for 5 min at 37 °C. Quench the digestion with 8 mL of the Plat-E cell medium, and pipette up and down to break up the clumps into single cells.
3. Collect the digested Plat-E cells into a 15-mL tube and centrifuge for 3 min at 300 $\times g$.
4. Resuspend the Plate-E cells in the Plat-E cell medium and count cell numbers.
5. Plate 8×10^6 cells in one gelatin-coated 100-mm dish in the Plat-E cell medium without any antibiotics (*see Note 15*).
6. Transfect Plat-E cells the next day (*see Note 16*) by using FuGENE® 6 system (*see Note 17*). Here is an example for transfection of a 100-mm dish, which produces 10 mL of retroviral supernatants of one reprogramming factor (*see Note 18*).
 - (a) For each retroviral vector of GMT factors, add 300 μ L of Opti-MEM I into a 1.5-mL Eppendorf tube; add 27 μ L Fugene® 6 in Opti-Mem, and mix gently by finger tapping. Incubate for 5 min at room temperature.
 - (b) Add 9 μ g of one retroviral vector DNA (i.e., pMXs-Gata4, -Mef2c, -Tbx5, or -dsRed) and mix thoroughly by finger tapping. Incubate for 15 min at room temperature.
 - (c) Add the DNA-FuGENE complex into the culture media dropwise and move the dishes back and forth gently to mix the DNA-FuGENE complex with the media in the dishes. Culture the transfected cells overnight.
7. In the morning of the next day after transfection, remove the spent medium (*see Note 19*), and add 10 mL of fresh pre-warmed Plat-E cell medium. Culture the transfected Plat-E cells for additional 24 h to produce high-titer viruses.

8. Harvest and filter retroviral supernatants through a Nalgene syringe filter (0.45-μm pore-size, SFCA-membrane) using a 10-mL sterile disposable syringe to remove the cell debris.
9. Add polybrene (final concentration 5 μg/mL) into the filtered virus-containing supernatant and mix gently by pipetting up and down. To make a retroviral reprogramming cocktail, add equal amount of retroviral supernatant of each factor (i.e., *Gata4*, *Mef2c*, and *Tbx5* in our study) and mix.

3.4 Production of iCMs from Mouse Fibroblasts by Retroviral Transduction of GMT

1. Aspirate the medium from the cultured FACS-purified fibroblasts from step 10 of Subheading 3.2. Add 0.5 mL of the fresh iCM-reprogramming medium with polybrene (5 μg/mL) into each well of fibroblasts.
2. Add 1.5 mL of the retroviral cocktail of the three factors into each well of fibroblasts, i.e., 0.5 mL retrovirus of each factor (*Gata4*, *Mef2c*, and *Tbx5*) (see Note 20).
3. Culture the cells overnight. After 24 h, remove the transduction medium (see Note 19) and add 2 mL fresh iCM-reprogramming medium into one well of a 6-well plate.
4. Change medium every 2–3 days. Examine the progress of iCM-reprogramming by checking for αMHC-GFP⁺ cells under a fluorescence microscope (Fig. 4a) (see Note 21).
5. Determine the reprogramming efficiency by FACS assay to quantitate the number of αMHC-GFP⁺ cells, or by quantification of cells positive for cardiac troponin-T (cTnT) via immunostaining (Fig. 4b).
6. Evaluate the reprogramming by examining expression of the cardiac enriched genes (e.g., α-actinin, cTnT) and the fibroblast-enriched genes at different time points after retroviral transduction. For example, αMHC-GFP⁺ and GFP⁻ cells can be purified by FACS sorting and used to profile gene expression of the reprogrammed iCMs by RNA-microarray or RNA-sequencing; activation of the cardiac genes and inactivation of the fibroblast-enriched genes (see Note 22) can be validated by qRT-PCR.
7. Conduct functional characterization of iCMs, such as Ca²⁺ transients, action potential and cell contraction, at 2–8 weeks after retroviral transduction (see Note 23).

4 Notes

1. Cardiac epigenetic reprogramming could be studied by using other transgenic mouse of cardiac lineage reporter, such as αMHC-mCherry transgenic mice (Stock No.: 021577) from The Jackson laboratory.

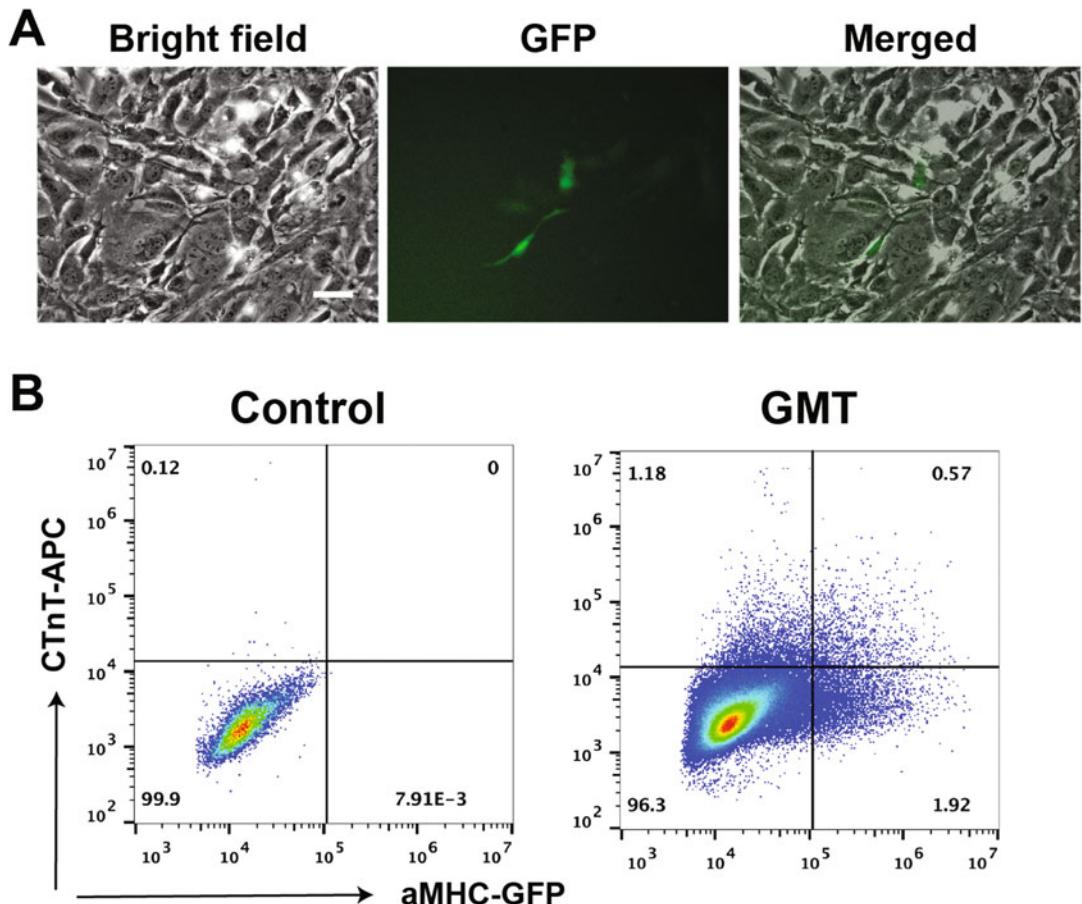


Fig. 4 (a) Representative pictures of reprogrammed α MHC-GFP $^+$ iCMs from neonatal cardiac fibroblasts 3 days after retrovirus infection of Gata4, Meft2c, and Tbx5 (GMT). Bar indicates 50 μ m. (b) FACS assay of cardiac troponin T (cTnT) and α MHC-GFP staining of reprogrammed neonatal cardiac fibroblasts 3 days after GMT retrovirus infection

2. In our experience, tissues from one heart of a neonatal mouse should be plated into one 60-mm dish and three hearts into one 100-mm dish; two tail-tip tissues, harvested at 0.5–0.75 cm in length, should be plated in one 100-mm dish; tissues from one ventricle of an adult mouse should be plated in two or three 100-mm dishes.
3. Keep tissues cold to minimize cell death or senescence. The harvested tissues should be used as soon as possible, but can be stored on ice for 2 h without obvious negative effect on fibroblast isolation.
4. Place the heart in one well of 24-well plate and the tail-tip from the same animal into the next well of the 24-well plate, so that α MHC-GFP-harboring tail-tips can be determined based on GFP expression in the heart from the same animal and will be used for fibroblast isolation.

5. It is critical to mince the tissues into small pieces so that the tissues can attach to cell culture vessels. Attachment is required for fibroblasts to emigrate from the isolated tissues. In our experience, it is more efficient to mince tissues without addition of media.
6. The amount of media depends on number of the collected α MHC-GFP-harboring tissues that are used for cell culture. For examples, we use 0.5 mL media for tissues of one neonatal heart that will be plated into one 60-mm dish, and 1.5 mL media for tissues of three neonatal hearts that will be plated into one 100-mm dish.
7. It is important to use a proper amount of media so that the media barely cover the surface of dish. Too much medium prevents attachment of the minced tissues onto the culture dishes.
8. Tissue attachment requires a minimum of 2 h. A period of 4 h of attachment is recommended.
9. It is very important to avoid disturbing the attached tissues. Do not add media directly upon tissue.
10. It is a good sign if the media become yellowish after 3 days of culture, suggesting that many fibroblasts have emigrated out from the attached tissues. Under a microscope, emigrated fibroblasts can be observed around the attached tissues.
11. Adult cardiac fibroblasts can be cultured for additional 3 days if needed.
12. It is not recommended to use the frozen or passaged fibroblasts for iCM-reprogramming.
13. To save the antibody used in Thy1-staining at **step 7**, it is fine to resuspend cells in 0.5 mL FACS solution but make sure no excess number of cells is used.
14. The ecotropic retroviruses, prepared from Plat-E cells, infect mouse cells only, and do not infect human cells.
15. It is important that the media of retrovirus preparation should not include any residual puromycin and blasticidin, which will kill fibroblasts when they are infected by the retroviral supernatants.
16. After overnight culture (~16 h), the culture of Plat-E cells should become >80% confluent for transfection. To improve the virus titer, it is recommended to refresh the Plat-E Cell Medium 1 h before transfection.
17. Other transfection reagents might be used for transfection. In our experience, FuGENE® 6 gives us the best yield of retrovirus.

18. For each batch of retrovirus preparations, dsRed viruses should be included to monitor the efficiency of transfection and virus preparation. For example, to determine the transfection efficiency, dsRed fluorescence should be observed in most of the Plat-E cells (>80%) in the pMX-dsRed packaging. dsRed retroviruses could be used for virus titration as needed. To save transfection reagents, retroviruses of pMX-dsRed can be prepared in a smaller 60-mm dish.
19. Dishes, pipettes, tips, and media, all of which possibly contain virus, must be treated by 10% bleach for >1 h before discarded. After overnight transfection, it is expected that Plat-E cells have produced retroviruses.
20. We routinely use 0.5 mL of viruses for each factor, but 0.3 mL of each viruses gave similar results in our experience. Our FACS assay showed that both 0.3 mL and 0.5 mL dsRed retroviruses could transduce >90% of fibroblasts without significant difference.
21. In our experience, α MHC-GFP⁺ cells can be observed at day 3 after transduction, suggesting a success of reprogramming; 1 week later, many α MHC-GFP⁺ cells should be observed. iCM-reprogramming failed if there are very few or no α MHC-GFP⁺ cells 7 days after retroviral transduction.
22. Cardiac muscle genes include *Actc1*, *Myh6*, *Ryr2*, *Myl7*, *Scn5a*, *Slc8a1*, *Myl2*, *Tnnt2*, *Pln*, *Kcna5*, *Kcnj2*, *Cacba1c*, *Gja1*, *Atp2a2*; fibroblast-enriched genes include *Colla1*, *Colla2*, *Col3a1*, *Vim*, *Posn*, *Fsp1*, *Fn*.
23. Fibroblasts infected by dsRed retroviruses should be included as a negative control in assays of α MHC-GFP⁺ iCM characterization.

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Chapter 4

Production of Cardiomyocytes by microRNA-Mediated Reprogramming in Optimized Reprogramming Media

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Abstract

There are currently no effective treatments to regenerate the heart after cardiac injury. Following cardiac injury, heart muscle cells, also known as cardiomyocytes, die in large numbers. The adult mammalian heart does not have the ability to replace these dead cardiomyocytes. In their place, fibroblasts invade the injury zone and generate a scar. The scar impairs cardiac function. An important approach to cardiac regeneration is direct cardiac reprogramming, whereby cardiac fibroblasts within the scar are directly converted into functional cardiomyocytes. Several laboratories have achieved direct cardiac reprogramming via overexpression of the cardiac transcription factors. In contrast, we utilize a combination of four miRNAs (miR-1, miR-133, miR-208, miR-499) that we call miR Combo. One common issue regarding direct cardiac reprogramming strategies is low efficiency. Recently, we have demonstrated that the efficiency of direct cardiac reprogramming is enhanced in the chemically defined reprogramming media.

Key words Reprogramming, Cardiac, Cardiomyocyte, MicroRNA, Fibroblast, Regeneration, Ascorbic acid, Selenium

1 Introduction

Following myocardial injury, cardiomyocytes die in significant numbers. These cardiomyocytes are not replaced as the adult mammalian heart lacks regenerative capacity [1]. In their place, fibroblasts invade the injury zone, replicate, and form fibrous scars [2]. These fibrous scars impair cardiac function. Over time, impaired cardiac function leads to heart failure. Converting fibroblasts within the fibrous scars into cardiomyocytes would be clinically beneficial as it may restore cardiac function. Cardiomyocytes can be generated from iPS cells [3]. However, while the generation of cardiomyocytes from iPS cells is relatively efficient, the resulting cardiomyocytes tend to be immature and may result in arrhythmia when they are implanted into the heart [3]. Moreover, any iPS cells contaminating the cardiomyocyte population can potentially develop into a tumor. In contrast to the iPS approach, a number

of laboratories have developed methods to convert fibroblasts directly into cardiomyocytes. One common method is the over-expression of transcription factors known to be involved in cardiac development [4, 5]. Alternatively, our laboratory has demonstrated direct reprogramming of fibroblasts into cardiomyocytes by a combination of four microRNAs (miR-1, miR-133, miR-208, and miR-499) that we call miR Combo [6–8]. When compared to iPS-derived cardiomyocytes, cardiomyocytes derived from direct cardiac reprogramming are mature. Due to their mature nature they do not induce arrhythmia. Irrespective of the method employed, converting fibroblasts directly into cardiomyocytes is relatively inefficient.

We have demonstrated that the efficiency of direct cardiac reprogramming is significantly increased by Insulin, Transferrin, Selenium, and Ascorbic acid (ITSA) [9]. Addition of these components to the culture medium enhances sarcomere formation and reduces the time taken for induced cardiomyocytes to appear. Importantly, the induced cardiomyocytes are mature. This method provides a useful way to generate mature cardiomyocytes in culture. These mature cardiomyocytes are potentially useful for testing drug cardiotoxicity as well as for cell transplantation studies.

In this chapter, we describe a detailed protocol for the direct reprogramming of fibroblasts into mature cardiomyocytes. Figure 1 outlines the major steps of the protocol. The procedure starts with transfection of fibroblasts with a cocktail of microRNAs called miR

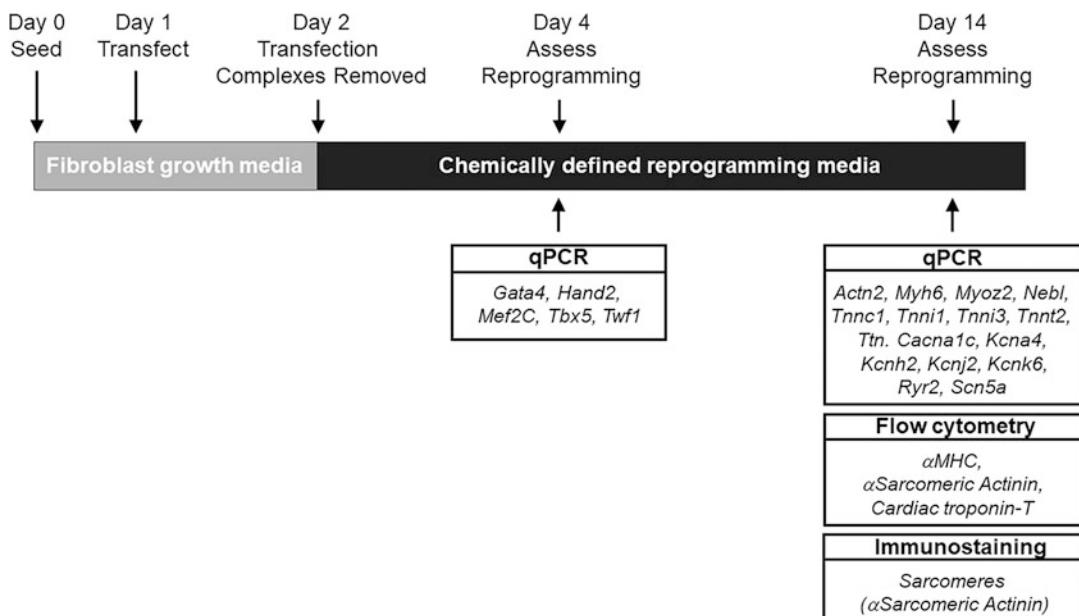


Fig. 1 Summary of protocols for reprogramming fibroblasts into mature cardiomyocytes with miR Combo. Arrows indicate time points at which the indicated steps are carried out

Combo. One day after transfection, the transfection complexes are removed and the cells are cultured in a chemically defined reprogramming media containing ITSA. After 3–4 days, the cells express cardiac commitment factors. By day 14, the cells display the features of mature cardiomyocytes. Sarcomeres are evident and the cells are spontaneously beating. This chapter will provide step-by-step procedures for: (1) isolation of neonatal cardiac fibroblasts (Subheading 3.1); (2) reprogramming fibroblasts into cardiomyocytes by miRNAs (Subheading 3.2); (3) evaluation of cardiac reprogramming by qPCR (Subheading 3.3); (4) evaluation of cardiac reprogramming by flow cytometry (Subheading 3.4); and (5) evaluation of cardiac reprogramming by immunofluorescence (Subheading 3.5).

2 Materials

2.1 Neonatal Cardiac Fibroblast Isolation

1. Digestion buffer: Digestion buffer—Hanks' Balanced Salt Solution (Calcium/Magnesium free) supplemented with Collagenase type II, 100 Units/mL.
2. Gelatin-coating solution: 0.2% gelatin in PBS.
3. Cell strainers (100 µm).

2.2 miR Combo

miR Combo is comprised of four miRNAs: miR-1; miR-133; miR-208; and miR-499 (*see Notes 1 and 2*). The sequences of the individual miRNAs in miR Combo are as follows:

miR-1: 5'UGGAAUGUAAAGAAGUAUGUAU3'.

miR-133a-3p: 5'UUUGGUCCCCUUCAACCAGCUG3'.

hsa-miR-208a-3p: 5'AUAAGACGAGCAAAAGCUUGU3'.

hsa-miR-499a-5p: 5'UUAAGACUUGCAGUGAUGUUU3'.

Prepare miR Combo with the supplied sterile DNase-/RNase-free water in a tissue culture hood.

To each vial of 5 nM miRNA, add 100 µL of the supplied sterile DNase-/RNase-free water. Incubate for 20 min at room temperature. Ensure all material is in solution by pipetting up and down >20 times. Combine the contents of the four vials into one vial. Mix thoroughly. The miR Combo is now ready for use (*see Notes 3 and 4*).

2.3 Cell Culture Media

1. *Fibroblast growth media (500 mL)*: 420 mL DMEM (4 mM L-glutamine, 4.5 g/L glucose, 1 mM sodium pyruvate, and 1.5 g/L sodium bicarbonate) (*see Note 5*); 75 mL Fetal Bovine Serum (*see Note 6*); 5 mL of 100× Penicillin/Streptomycin solution. Mix thoroughly by inversion and Store media at 4 °C. Avoid excessive exposure to light and use within 1 month.

2. *Chemically defined reprogramming media (500 mL)*: 245 mL DMEM; 245 mL F12; 2% Bovine Serum Albumin (*see Note 7*); 5 mL Insulin-Transferrin-Selenium (10 mg/L Insulin; 5.5 mg/L Transferrin; 6.7 µg/L Sodium Selenite); 5 mL Penicillin/Streptomycin; and 22 mg of Ascorbic acid (250 µM). Filter media with a 0.22 µm sterile filter. Store media at 4 °C. Avoid excessive exposure to light and use within 1 month (*see Note 8*).

2.4 Tissue Culture and Transfection

1. Trypsin (0.05%).
2. Heat-sterilized PBS (1×) pH 7.4.
3. Gelatin (2%).
4. Dharmafect-I.

2.5 qPCR

1. RNA-extraction kit.
2. High capacity cDNA reverse transcription kit.
3. 0.2 mL PCR 8-tube strips.
4. TaqMan Gene Expression Master Mix.
5. MicroAmp Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL.
6. Microseal(R) "B" Adhesive Seals.
7. TaqMan probes: Twf1; Gata4; Hand2; Mef2C; Tbx5; Actn2; Myh6; Myoz2; Nebl; Tnncl; Tnnil; Tnni3; Tnnt2; Ttn; Cacna1c; Kcna4; Kcnh2; Kcnj2; Kcnk6; Ryr2; Scn5a; Gapdh (*see Notes 9 and 10*).

2.6 Flow Cytometry

1. FACS buffer: 1× PBS, 2 mM EDTA, 5% BSA, 0.2% saponin. Store 4 °C.
2. Primary Antibodies: αMHC; αSarcomeric Actinin; FITC-conjugated cardiac troponin-T (*see Note 11*).
3. Secondary Antibodies: Anti-mouse APC; Anti-rabbit Alexa-Fluor 488.
4. Control IgGs: Mouse IgG1 FITC; Mouse IgG1 APC; Rabbit IgG FITC.

2.7 Immunostaining

1. Antibody buffer: 1× PBS, 1% BSA, 0.3% Triton X-100, pH 7.4. Store 4 °C.
2. α-Sarcomeric actinin antibody (*see Note 12*).
3. Goat Anti-mouse 594 nm secondary antibody.

3 Methods

3.1 Isolation of Neonatal Cardiac Fibroblasts [7]

1. Coat T25 flasks with 0.2% gelatin at 37 °C in an incubator for at least 30 min prior to use. For one litter of mice, coat four T25 flasks.
2. Harvest hearts from 1-day-old neonatal mouse pups (*see Note 13*): first disinfect the pups with 70% ethanol, decapitate, and cutting through the frontal rib cage to reveal a clear view of the hearts and lungs (*see Note 14*).
3. Excise the hearts from mice and place the excised hearts in a 35-mm dish containing PBS.
4. Remove atria and any other attached non-cardiac tissue.
5. Move the ventricles immediately to digestion buffer.
6. Once in digestion buffer, cut the tissue into very small pieces using a sterile razor blade (*see Note 15*).
7. Incubate the minced tissue in 2 mL of the same digestion buffer for 10 min at 37 °C.
8. At the end of the incubation, pass the lysate through a cell strainer (100 µm) and collect the flow-through in a 50-mL Falcon tube.
9. Add 10 mL of fibroblast growth media to the flow-through to deactivate collagenase.
10. Centrifuge cell mixture at $400 \times g$ for 5 min at room temperature.
11. Aspirate to remove fibroblast growth medium and deactivated collagenase.
12. Resuspend cell pellet in 10 mL fibroblast growth medium.
13. Immediately before seeding cells, aspirate gelatin solution from the T25 flasks.
14. Seed the cells into the gelatin-coated T25 flasks (2.5 mL per flask) and add 7.5 mL fibroblast growth media.
15. Change the media at 24 h.
16. Refresh media every 48 h until cells reach confluence.
17. Once the cells have reached confluence (*see Note 16*), coat four T75 flasks with 0.2% gelatin at 37 °C in an incubator for at least 30 min prior to use.
18. Wash the cell monolayer twice with sterile PBS.
19. Add 1.5 mL 0.05% trypsin to each T25 flask and incubate for 5 min at 37 °C (*see Note 17*).
20. Add 4 mL fibroblast growth media to each T25 flask.
21. Combine the cells from each flask in one 50-mL Falcon tube.

22. Centrifuge cell mixture at $400 \times g$ for 5 min at room temperature.
23. During the centrifugation step, remove the gelatin solution from the T75 flasks.
24. Once the centrifugation step has finished, remove the fibroblast growth medium and deactivated trypsin from the 50-mL Falcon tube.
25. Resuspend the cell pellet in 10 mL of fibroblast growth medium.
26. Add 2.5 mL cells to one gelatin-coated T75 flask.
27. Add 7.5 mL of fibroblast growth media to each T75 flask.
28. Refresh media every 2 days.
29. The fibroblasts are ready to use when the cells have reached 70–80% confluence (*see Note 18*).

3.2 Cardiac Reprogramming by microRNA

1. When the P1 fibroblasts are ready for use (*see step 29* in Subheading 3.1), warm media, 2% gelatin, and PBS in a 37 °C water bath.
2. Prepare 0.2% gelatin in sterile 1× PBS solution.
3. Add gelatin solution to 12-well plate (0.5 mL per well) and incubate at 37 °C in an incubator for >10 min prior to use.
4. Immediately before seeding cells, completely aspirate gelatin solution.
5. Wash Passage 1 fibroblasts with 20 mL of sterile 1× PBS. Remove PBS and add 2 mL of 0.05% trypsin. Rotate flask to ensure the entire cell layer is covered with trypsin.
6. Incubate at 37 °C in an incubator for no more than 5 min. Add 10 mL of fibroblast growth media per flask to quench trypsin.
7. Transfer contents of the flask into a sterile 50-mL tube. Centrifuge the cells at $400 \times g$ for 5 min.
8. Following centrifugation, remove media and resuspend the cell pellet in 10 mL of fibroblast growth media.
9. Count cells and seed at 22,500 cells per well of a 12-well plate (*see Note 19*) in 1 mL total volume per well. Cells will be ready for transfection 24 h after seeding (*see Note 20*).
10. For transfection, set up two tubes. In tube 1, add 1.5 µL miRNA stock and 148.5 µL serum-free DMEM media for each well of a 12-well plate. In tube 2, add 1.5 µL Dharmafect-I and 148.5 µL serum-free DMEM media for each well of a 12-well plate. After incubation for 5 min at room temperature, combine tubes 1 and 2. Swirl by hand to mix. Incubate for 20 min at room temperature.

11. Immediately prior to adding the transfection complexes, remove media from the wells. Add transfection complexes to wells and then add 450 µL of fibroblast growth media (*see Note 21*).
12. Remove transfection complexes after 24 h. Culture cells in chemically defined reprogramming media (1 mL per well) for the duration of the experiment. Refresh media every 2 days.
13. The efficiency of miR Combo transfection is determined 3 or 4 days post transfection by measuring the expression of the miR-1 target, Twf1 (*see Fig. 1*).
14. Four days post transfection, we evaluate mRNA levels of cardiac transcription factors by qPCR (*see Fig. 1*).
15. Fourteen days post transfection, we evaluate mRNA levels of sarcomere components and cardiomyocyte-specific ion channels by qPCR (*see Fig. 1*). We also evaluate sarcomere protein levels by flow cytometry and immunostaining (*see Fig. 1*).

3.3 Evaluation of Cardiac Reprogramming by qPCR

1. Aspirate media from the cells (*see Note 22*) and add 350 µL of RNA lysis buffer into each well. The cells are removed from the culture plate by scraping with a pipette tip.
2. Remove genomic DNA via centrifugation at room temperature (*see Note 23*) in a Spin-Away filter column (10,000 × g for 1 min).
3. Add 350 µL of molecular biology grade ethanol to the eluate and mix well via pipetting.
4. Add mixture to a Zymo-Spin column. Centrifuge at 10,000 × g for 1 min. Discard flow through.
5. Add 400 µL RNA-prep buffer to each column. Centrifuge at 10,000 × g for 1 min. Discard flow-through.
6. Add 700 µL of Wash Buffer to each column. Centrifuge at 10,000 × g for 1 min. Discard flow-through.
7. Add 400 µL of Wash Buffer. Centrifuge at 20,000 × g for 2 min. Place column in a sterile 1.5-mL tube. Add 30 µL of the supplied DNase/RNase-free water to each column (*see Note 24*). Stand for 1 min and then centrifuge at 10,000 × g for 1 min. Discard the column, and RNA is ready to use (*see Note 25*).
8. Total RNA (50–100 ng; *see Note 26*) is converted to cDNA using a high capacity cDNA reverse transcription kit.
9. For each sample, set up the following reaction in a 250 µL capacity DNase/RNase-free PCR tube: 4 µL 10× RT buffer; 2 µL dNTP mix; 4 µL 10× Random Primers; 2 µL MultiScribe Reverse Transcriptase; x µL RNA (250 ng–1 µg); add DNase/RNase-free water to a final volume of 40 µL (*see Note 27*).

10. The cDNA reaction is as follows: 1 cycle 25 °C for 10 min; 1 cycle 37 °C for 120 min; 1 cycle 85 °C for 10 min; indefinite hold at 4 °C. The cDNA is now ready to use for qPCR.
11. qPCR is performed with an Applied Biosystems StepOne Plus instrument in a 96-well plate format. Each qPCR reaction is set up as follows: 1 µL cDNA; 1 µL qPCR primer; 10 µL TaqMan Gene Expression Master Mix; 8 µL DNase/RNase-free water.
12. The PCR reaction is as follows: 1 cycle 50 °C for 2 min; 1 cycle 95 °C for 10 min; 40 cycles of 95 °C for 15 s; and 60 °C for 1 min.
13. Calculate expression levels relative to the housekeeping gene GAPDH. First ΔCt is calculated: $\Delta Ct = Ct$ (gene of interest) – Ct (GAPDH). Expression level is calculated from the ΔCt value: Expression = $2^{-\Delta Ct}$ (*see Note 28*).

3.4 Evaluation of Cardiac Reprogramming by Flow Cytometry

1. Wash cell monolayer twice with PBS (2 mL per well per wash).
2. Add 0.5 mL (12-well plate) or 1 mL (6-well plate) of 0.05% trypsin to each well.
3. Incubate for 5 min at 37 °C to detach the cells from the tissue culture plate (*see Note 29*).
4. Trypsin is neutralized via the addition of 500 µL complete serum. Remove a 40 µL of the cell suspension for cell counting. Pellet remainder of the cells by centrifugation at $400 \times g$ for 5 min at room temperature.
5. Aspirate the trypsin and serum solution and resuspend the pellet in 100 µL ice-cold 4% paraformaldehyde (*see Note 30*).
6. Incubate for 15 min at 4 °C.
7. Add 1 mL ice-cold FACS buffer and pellet cells by centrifugation at $800 \times g$ for 5 min at 4 °C.
8. Aspirate the FACS buffer and paraformaldehyde solution, then resuspend the cell pellet in 100 µL ice-cold FACS buffer.
9. Add antibodies at 0.4 µg/ 10^6 cells (*see Note 31*).
10. Incubate at 4 °C for 1 h with constant agitation.
11. Add 1 mL ice-cold FACS buffer and pellet cells by centrifugation at $800 \times g$ for 5 min at 4 °C.
12. Aspirate the FACS buffer and resuspend pellet in 1 mL ice-cold FACS buffer. Pellet cells by centrifugation at $800 \times g$ for 5 min at 4 °C.
13. Repeat **step 12**.
14. If the antibody is conjugated with a fluorophore, proceed to **step 15**. If the antibody is not conjugated with a fluorophore; resuspend pellet in 100 µL FACS buffer and add appropriate conjugated secondary antibody (0.4 µg/ 10^6 cells). Incubate at 4 °C for 1 h with constant agitation and repeat **steps 11–13**.

15. Resuspend pellet in 250 μ L FACS buffer and perform flow cytometry on instrument of choice (*see Notes 32 and 33*).
16. Analyze flow cytometer data (*see Note 34*).

3.5 Evaluation of Cardiac Reprogramming by Immunofluorescence

1. Wash the cell monolayer twice with 1 \times PBS (1 mL per well).
2. Add 0.5 mL of 2% paraformaldehyde and incubate at room temperature for 10 min.
3. Aspirate the fixation buffer and wash the cells twice with 1 \times PBS (1 mL per well) (*see Note 35*).
4. Block by incubating the cells in antibody buffer (0.5 mL per well) for 1 h at room temperature.
5. Replace antibody buffer with fresh antibody buffer; 300 μ L per well.
6. Add primary antibodies at their indicated dilution and incubate overnight at 4 °C with gentle agitation.
7. Aspirate the antibody solution and wash the cells three times with antibody buffer (1 mL per well). Each wash is 5 min in duration at room temperature with gentle agitation.
8. To each well, add 500 μ L antibody buffer and 0.5 μ L Alexa-Fluor-conjugated secondary antibody.
9. Incubate for 1 h at room temperature with gentle agitation. Thirty minutes before the end of the incubation, add DAPI to a final concentration of 1 μ g/mL.
10. Aspirate the antibody solution and wash the cells three times with antibody buffer (1 mL per well). Each wash is 5 min in duration with gentle agitation at room temperature.
11. After the final wash, aspirate the antibody buffer.
12. Add 1 mL of PBS to each well. If the plates are to be assessed later, store plates at 4 °C. Make sure to add penicillin/streptomycin (1 \times final concentration) to prevent bacterial growth during the storage.
13. Photograph cells with a fluorescent microscope (*see Note 36*).

4 Notes

1. It is important to note that the efficiency of reprogramming is very dependent upon reagent quality. We have found that reagents with the same name from different suppliers typically differ greatly in their quality. As such, we have spent considerable time identifying the optimal reagents for our studies. Researchers wishing to repeat our studies in their own laboratories would be well advised to acquire the reagents from the

same sources we use. To that end, to help researchers achieve successful reprogramming experiments, the sources and catalog numbers of the reagents we use are provided in this section.

2. Pre-miRNAs from ThermoFisher offer the most reliable results. The catalog numbers are: hsa-miR-1: PM10617; hsa-miR-133a-3p: PM10413; hsa-miR-208a-3p: PM10677; hsa-miR-499a-5p: PM11352.
3. miR Combo can be stored at -20 or -80 °C. Use within 3 months. Repeated freeze-thaw does not influence results.
4. We use a non-targeting miRNA (negmiR) as a control (ThermoFisher Cat no. AM17110). To prepare negmiR, add 100 μ L sterile DNase-/RNase-free water to each vial of 5 nM. Incubate for 20 min at room temperature. Ensure all material is in solution by pipetting up and down >20 times. NegmiR can be stored at -20 or -80 °C. Use within 3 months.
5. We have found that DMEM quality differs between manufacturers. Through testing, ATCC DMEM (Cat no. 30-2002) was found to be most optimal for reprogramming.
6. Serum has a significant effect on reprogramming. When our supply of serum is exhausted, we batch test 3–4 different fetal bovine sera and evaluate the effects on reprogramming. Once we have identified a serum batch that does not interfere with miR Combo reprogramming, we purchase 20–30 500-mL bottles of that batch and store them at -20 °C. Prior to use, the serum bottle is thawed at 4 °C for 2–3 days and aliquoted into 50-mL tubes (37.5 mL per tube) and stored at -20 °C until needed.
7. Serum can be substituted for bovine serum albumin.
8. While the chemically defined reprogramming media can be used for up to 1 month, we find that reprogramming efficiency is greatly enhanced when the chemically defined reprogramming medium is made immediately prior to use.
9. We use validated TaqMan primers from ThermoFisher for our assays. For Twfl, the assay ID number is Mm00725968_s1; Mm00484689_m1 for Gata4; Mm00439247_m1 for Hand2; Mm01340842_m1 for Mef2C; Mm00803518_m1 for Tbx5; Mm00473657_m1 for Actn2; Mm00440359_m1 for Myh6; Mm00469639_m1 for Myoz2; Mm00503886_m1 for Nebl; Mm00437111_m1 for Tnncl; Mm00502426_m1 for Tnni1; Mm00437164_m1 for Tnni3; Mm01290256_m1 for Tnnt2; Mm00621005_m1 for Ttn; Mm00437917_m1 for Cacna1c; Mm01336166_m1 for Kcna4; Mm00465377_mH for Kcnh2; Mm00434616_m1 for Kcnj2; Mm01176312_g1 for Kcnk6; Mm00465877_m1 for Ryr2; Mm01342518_m1 for Scn5a; and Mm99999915_m1 for Gapdh (*see Note 10*).

10. 18S can also be used as a housekeeping gene. However, the cDNA has to be diluted as 18S expression is very high. Beta-Actin is not suitable as it is an miR-1 target.
11. Antibodies from commercial vendors are of various quality. We recommend the following antibodies: α MHC (Abcam Cat no. ab15); α Sarcomeric Actinin (Abcam Cat no. Ab68167); FITC-conjugated cardiac troponin-T (Abcam Cat no. ab105439).
12. Commercial antibodies differ greatly in their quality. For immunostaining, we only recommend the α -sarcomeric actinin antibody from Sigma (Sigma Cat no. A7811).
13. Reprogramming efficiency decreases with age. Fibroblasts from 1-day-old mice give the highest reprogramming efficiency.
14. If these steps are performed rapidly, the hearts can be easily distinguished via its pumping action. We typically perform a “shelling of a pea” approach: once the thoracic cage has been opened, we pinch the back of the pup, forcing the heart out of the ribcage.
15. Mincing of the tissue in a small volume of digestion buffer (0.25 mL) permits easier handling.
16. Fibroblasts are passaged when they reach confluence 4–5 days after isolation. The cells are relatively small and tend to grow in colonies. We find that if the cells take longer than 5 days to reach confluence, or if they appear senescent, they reprogram poorly.
17. Trypsinization for more than 5 min significantly impairs reprogramming. Ignore any cells that remain attached to the flasks after agitation.
18. Do not let the cells become confluent at this stage.
19. Seeding at 27,000 cells per well of a 12-well plate improves sarcomere maturation.
20. Transfecting fibroblasts with microRNAs less than 24 h after seeding the cells results in poor reprogramming efficiency.
21. Transfection efficiency is improved if the transfection complexes are added to the cells before the fibroblast growth media.
22. The mRNA levels of the cardiomyocyte-specific transcription factors peak before mRNA levels of cardiomyocyte-specific sarcomere and ion channel genes. Therefore, we assess expression levels of cardiomyocyte-specific transcription factors such as Gata4, Hand2, Mef2c, and Tbx5 4 days after transfection. Expression of cardiomyocyte-specific sarcomere genes (Actn2, Myh6, Nebl, Tnni3, and Ttn) and cardiomyocyte-specific ion channels (Cacna1c, Scn5a, and Ryr2) are assessed 14 days after transfection.

23. Centrifugation at 4 °C results in reduced RNA yield.
24. Spin columns tend to have high static charges and to ensure effective recovery of the RNA we place the pipette tip 1 mm above the column membrane in the center of the spin column.
25. RNA can be stored briefly at –20 °C (1–2 days). For longer term storage, –80 °C is necessary. There is some loss of RNA during storage, and ideally RNA should be used within 1 week after isolation.
26. Fold changes as determined by qPCR are insensitive to the amount of cDNA; as such, exact titration of the RNA amount used in the cDNA reaction is unnecessary.
27. It is important to add the components in the order listed.
28. We typically perform qPCR with a technical duplicate or triplicate. These technical duplicates/triplicates are not a single cDNA reaction in two or three qPCR reactions. Instead, they are a single qPCR from two or three wells from the same batch of fibroblasts. We find that this is more informative of the variance in each experiment.
29. While it is possible to carry out flow cytometry with cells from a 12-well plate (1 well per sample), we find that 6-well plates (1 well per sample) are more reliable in providing enough cells for the experiment. To seed a 6-well plate for miRNA transfection, seed the wells with 40,000 cells per well 1 day prior to miRNA transfection.
30. The paraformaldehyde must be made in a pH 7.4 buffer to prevent cell rupture. We typically use 1× PBS.
31. We carry out both isotype and no staining controls. Isotype controls can give high backgrounds, and it is sometimes necessary to change the supplier or to repeat the experiment by first blocking the cells. To block the cells, we incubate the cells in ice-cold FACS buffer for 1 h at 4 °C prior to the addition of the antibody. We incubate cells with antibody for no more than 1 h as we have found that longer incubations give rise to higher backgrounds.
32. Ideally, use a digital flow cytometer versus an analog cytometer. The former will allow for software-based compensation; whereas the latter can only support manual compensation. Software-based compensation is more accurate.
33. Rather than wasting precious samples, we set up an additional unstained sample for the sole purpose of setting the voltages for each channel. The ideal voltage places the cells in the first decalog. The cells should not be bunched on an axis. If the cells are bunched on an axis, adjust the voltage. Once the voltages are set, it is worth quickly running a stained sample to ensure that positively labeled cells are not off the top end of the axis.

34. Stringent gating is preferred. True positive cells are typically at least one decalog above background.
35. It is possible to store the plates at this point. The important point is to limit bacterial growth. To that end, we store our plates at 4 °C in a 1× PBS solution containing 1× Penicillin-Streptomycin. Plates can be stored for up to 1 month.
36. Background with α-sarcomeric actinin and α-myosin heavy antibodies can be quite high. When illuminated by the laser, true positive cells are intense. Despite their brightness, they can be hard to find and it is good practice to scan the entire well. Higher magnifications are necessary to visualize sarcomeres. We scan the well at 10× magnification and when we find a positive cell we increase the magnification to 40× to image sarcomeres.

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Chapter 5

Direct Reprogramming of Human Fibroblasts into Induced Neural Progenitor Cells Using Suicide Gene Embodied Episomal Vectors for Rapid Selection of Exogenous DNA-Free Cells

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Abstract

Direct neural reprogramming involves a rapid conversion of somatic cells into neural cells without passing through the intermediate pluripotent stage. This phenomenon can be mediated in the starting somatic cells by the introduction of lineage-specific master transcription factors or by pluripotency factors routinely used in iPS cell generation. In the latter process known as Pluripotency factor-mediated Direct Reprogramming (PDR), the pluripotency factors are used to elicit epigenetic changes producing a permissive state in the starting cells which are then driven to the neural lineages by simple manipulations of the culture conditions. When genes are exogenously introduced to achieve such conversion, their persistent expression after completion of the reprogramming can affect the properties of the resulting cells. Here, we describe a robust method for direct neural reprogramming using the episomal vectors that incorporate a suicide gene *scFCYI* (encoding cytosine deaminase) that allows rapid and efficient generation of a homogenous population of transgene-free human-induced neural progenitor cells (hiNPCs). The resulting NESTIN⁺/PAX6⁺/CDH2⁺ hiNPCs can be expanded and cryopreserved and can be further differentiated into neurons and glia.

Key words Direct reprogramming, Direct conversion, Cell fate change, Induced neural stem cell, Induced neural progenitor, Episomal vector, Cytosine deaminase, Suicide gene

1 Introduction

One of the remarkable discoveries in modern biology is the considerable plasticity exhibited by the terminally differentiated cells. It has been proven that the fates of the terminally differentiated cells can be changed, even across the three germ-layer barriers, by forced expression of a handful of reprogramming factors. This property has been first exploited to generate human-induced pluripotent stem cells (hiPSCs) [1]. More recently, several laboratories reported direct reprogramming (also known as direct conversion or lineage reprogramming) of human somatic cells to various tissue-specific

cells or progenitors using methods that bypass the intermediate pluripotent stage [2–4]. We and others have reported the use of lineage-specific master transcription factors in direct reprogramming of human fibroblasts to functional neurons (hiNs) under the defined conditions [2, 5]. Given that the yield of direct neuronal conversion will always be limited by quantity of the starting cells, which in turn can limit its application, we and others developed methods for direct conversion of somatic cells to neural progenitor cells (hiNPCs) that can be expanded, preserved, and terminally differentiated into neurons and glia [4, 6].

To generate hiNPCs from the dermal fibroblasts, we episomally introduced the pluripotency factors (“Yamanaka factors”), used in iPSC reprogramming, to elicit epigenetic changes in the starting cells that allow them to attain a “permissive” state for conversion to another type of cell fates. We then took advantage of the instructive capacity of signaling molecules and culture conditions to further drive these cells to hiNPCs. This pluripotency factor-mediated direct reprogramming (PDR) is very rapid and robust and can be easily adapted by any laboratory with experience in iPSC reprogramming [7–9]. Recently, hiNPCs generated by PDR from somatic cells of the Parkinson’s disease patients was shown to faithfully model some aspects of the disease [8]. Moreover, PDR has an added advantage that the same set of reprogramming factors and the same type of starting cells can be used to generate multiple cell lineages, through simple culture manipulations, without passing through the intermediate pluripotent state [5, 10].

A potential challenge to the original PDR approach is the possibility that the properties of the resulting cells may be adversely affected by sustained expression of the pluripotency factors or by rare integration of the episomal vectors into the host genome. To completely remove the episomal vectors, others have used continuous passaging and selection of the reprogrammed cells in over 2 months [11]. Such long process is an obstacle to the applications of direct reprogramming. We had shown that the simple introduction of a suicide gene *scFCYT1*, (encoding cytosine deaminase (CD)), to the reprogramming vectors can achieve complete removal of the population of cells that continue to express the transgenes, within 14 days post reprogramming [12]. When these cells are exposed to 5-fluorocytosine (5-FC), the activity of CD from the transgene converts the compound to the toxic product 5-fluorouracil (5-FU) which kills the CD-harboring cells. The resulting pool of the reprogrammed cells will be virtually devoid of any transgene. Here, we describe hiNPC generation by the PDR method using the CD-containing episomal vectors (CD-vector). In principle, the same approach can be extended to other lineage conversion protocols utilizing the PDR strategy to generate the transgene-free end products.

2 Materials

2.1 Human Fibroblasts Culture

1. Human fibroblast culture medium (hFM): Minimum Essential Media (MEM) with L-glutamine containing 10% fetal bovine serum (FBS), 0.1 mM non-essential amino acids, and 1 mM sodium pyruvate. The hFM is sterilized using a 0.22- μm polyethersulfone (PES) membrane filter unit and stored in the dark at 4 °C for up to 2 weeks.
2. Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium. DPBS is stored at room temperature.
3. 0.05% Trypsin-EDTA (T/E). The T/E is stored at -20 °C. One working aliquot can be stored at 4 °C for up to 2 weeks.

2.2 Generation of hiNPCs

2.2.1 Induction of hiNPC Reprogramming by Electroporation with CD-Episomal Vectors

1. Reprogramming vectors: pCXLE-hOCT4-CD, pCXLE-hSK-CD, and pCXLE-hUL-CD. All CD-vectors were generated by insertion of the yeast *scFCY1* (CD) gene and the pluripotency genes into the pCXLE-gw plasmid by Gateway cloning.
2. Reprogramming Medium-Neural (RepM-N) medium: RepM-N medium is composed of 1:1 mixture of Advanced Dulbecco's Modified Eagle Medium/Ham's F-12 (Advanced DMEM/F12) and Neurobasal medium. To this mixture, add 0.05% bovine serum albumin, 1× N2, 1× B27 minus Vitamin A, 2 mM Glutamax, and 0.11 mM β -mercaptoethanol are supplemented. The RepM-N is sterilized using a 0.22- μm PES membrane filter unit and stored in the dark at 4 °C for up to 2 weeks.
3. hiNPC medium: RepM-N supplemented with 3.0 μM CHIR99021, 0.5 μM A83-01, and 10 ng/mL human Leukemia Inhibitory Factor (hLIF). The hiNPC medium is stored in the dark at 4 °C for up to 2 weeks.
4. Geltrex (or Matrigel) solution for coating of culture dishes: Geltrex stock is thawed at 4 °C, and divided into 2 mg/tube aliquots and then stored at -80 °C. To make the working Geltrex solution, one tube of Geltrex stock aliquot is thawed at 4 °C for overnight, and then diluted with 12 mL cold DMEM/F12 medium.
5. Accutase: Accutase is stored at -20 °C. One working aliquot can be stored at 4 °C for up to 2 months.
6. 0.4% Trypan blue solution.
7. NEPA21 Super electroporator with electroporation cuvette (NEPA gene) or a similar device.

2.2.2 Selection and Isolation of the Successfully Reprogrammed hiNPC Cells

1. hiNPC medium: RepM-N supplemented with 3.0 µM CHIR99021, 0.5 µM A83-01, and 10 ng/mL human Leukemia Inhibitory Factor (hLIF). The hiNPC medium is stored in the dark at 4 °C for up to 2 weeks.
2. DPBS: *see item 2* of Subheading **2.1**.
3. Geltrex (or Matrigel) solution for coating of culture dishes: *see item 4* of Subheading **2.2.1**.
4. 23-G PrecisionGlide needle.
5. Stereoscopic microscope.

2.3 Removal of CD-Episomal Vectors and Copy Number Detection

1. 5-FC solution: Dissolve 5-FC powder to a final concentration of 50 mM in distilled water (D.W.) and store the solution at –20 °C.
2. DirectPCR Lysis Reagent (Viagen). This product is stable for up to 12 months at 4 °C.
3. Proteinase K solution: A 20 mg/mL proteinase K solution retains activity for at least 2 years when stored at –20 °C.
4. pCXLE-hFbx15-cont2 (Addgene, plasmid No. 27081): Control plasmid for copy number calculation.
5. iQ SYBR green supermix. This product contains SYBR Green I dye, DNA polymerase, dNTPs (0.4 mM each), 6 mM MgCl₂, 40 mM Tris–HCl (pH 8.4), 100 mM KCl, 20 nM fluorescein, and stabilizers. This product is stable for up to 6 months when stored at 4 °C.
6. Forward and reverse PCR primers for *FBXO15* and *EBNA1*. *FBXO15* forward (60 °C): GCC AGG AGG TCT TCG CTG TA; *FBXO15* reverse: AAT GCA CGG CTA GGG TCA AA; *EBNA1* forward (60 °C): AGA CAC ATC TGG ACC AGA AGG CTC; *EBNA1* reverse: TGT TCC ACC GTG GGT CCC TTT G.
7. C1000 thermal cycler (Bio-Rad) or a similar device.
8. 7500 Fast real-time PCR system (Applied Biosystems) or a similar device.

2.4 Characterization of hiNPCs

2.4.1 Immuno-cytochemistry (ICC)

1. Fixative solution: 4% paraformaldehyde. Mix 10 mL 16% paraformaldehyde, 4 mL 10× DPBS, and 26 mL D.W. This solution is stored at 4 °C for up to 1 month.
2. 3% bovine serum albumin (BSA) solution: Dissolve BSA fraction V to a final concentration of 3% in DPBS, divide the solution into 10 mL working aliquots, and store the aliquots at –20 °C.
3. Permeabilization and blocking (P&B) solution: 0.3% Triton X-100 in 3% BSA solution.

4. Primary antibodies: Mouse TUJ1 antibody, rabbit anti-GFAP, rabbit anti-PAX6, mouse anti-Ki-67, mouse anti-PLZF, and rabbit anti-ZO1, rabbit anti-CDH2 (N-Cadherin).
5. Secondary antibodies: Donkey anti-mouse IgG Alexa Fluor 488 (1:500), donkey anti-rabbit IgG Alexa Fluor 488 (1:500), and donkey anti-mouse IgG Alexa Fluor 594 (1:500).
6. Hoechst33342 solution: Dissolve Hoechst33342 to a final concentration of 10 mg/mL in D.W. and store at -20 °C.
7. Evos FL Auto 2 fluorescence microscope (Thermo Fisher Scientific) or a similar device.

2.4.2 Differentiation of hiNPCs

1. hiNPC differentiation (ND) medium: Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F12) medium containing 1× B27 minus Vitamin A, 50 µg/mL 2-phospho-L-ascorbic acid, 20 ng/mL BDNF, 20 ng/mL GDNF, and 0.5 mM dbcAMP. The ND medium is sterilized using a 0.22-µm PES membrane filter unit, and then stored in the dark at 4 °C for up to 2 weeks.
2. Poly-L-ornithine solution: This product is stable for up to 2 years from the production date when stored at 4 °C.
3. Laminin solution: Dilute 1 mg/mL laminin in DPBS without calcium and magnesium to a final concentration 3.3 µg/mL immediately before use. Do not vortex the laminin solution (*see Note 1*).
4. DPBS: *see item 2* of Subheading 2.1.
5. 0.4% Trypan blue solution.

3 Methods

3.1 Preparing Human Fibroblasts for hiNPC Reprogramming

1. Plate human fibroblasts in 10 mL hFM in a 100-mm tissue culture-treated dish. Adjust the plating density to achieve between 2.5×10^6 and 3.5×10^6 cells on the day of transfection (*see Note 2*).
2. Replace the culture medium with fresh prewarmed hFM every other day.

3.2 Direct Reprogramming of Fibroblasts to hiNPCs

A schematic overview of the hiNPC direct reprogramming protocol is provided in Fig. 1.

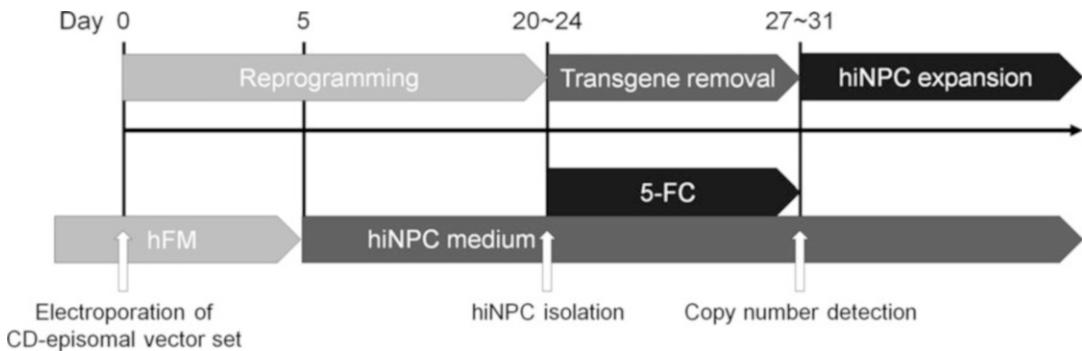


Fig. 1 Schematic representation of the procedure for direct reprogramming of hiNPC and production of cells devoid of transgenes. The method described here involves a three-stage process spanning across approximately 1 month. The processes include introduction of vectors into fibroblasts and then providing appropriate culture conditions that convert them into hiNPCs over a period of 20 days. The transgenes are then removed, and the resulting cultures are expanded to produce transgene-free hiNPCs. The top boxes provide information on stage-specific processes and the bottom boxes describe the key reagents and media used

*3.2.1 Delivery
of Reprogramming Factors
by Electroporation
of the CD-Episomal Vectors*

1. (Day -1), Transfer a Geltrex aliquot from a deep-freezer into a refrigerator and completely thaw the aliquot overnight.
2. (Day 0), Make the working Geltrex solution, then add 1 mL of the solution to each well of a 6-well plate. Coat a total of three wells. Coat the plate by incubation at 37 °C for at least 1 h.
3. Remove the culture medium from a nearly confluent 100-mm dish of human fibroblasts, then wash one time with 10 mL DPBS. Aspirate the used DPBS.
4. Add 2 mL 0.05% T/E and incubate the plate at 37 °C for 5 min.
5. Add 4 mL hFM to inactivate trypsin.
6. Gently pipet the cell solution to generate a single-cell suspension and collect the cell suspension in a 15-mL tube.
7. Centrifuge at $150 \times g$ for 5 min at room temperature.
8. Remove the supernatant carefully and add 5 mL fresh hFM.
9. Thoroughly resuspend the cells by pipetting up and down using a 1-mL pipette without producing air bubbles.
10. Count the live cells using trypan blue staining and a hemacytometer. Alternatively, one can use automated systems like Countess (Thermo Fischer) or NC-200 (Chemometec).
11. In a NEPA21 Super electroporator, set the poring pulse (We set the parameters to 170 V, 5 ms length, 50 ms interval, and two-times) and transfer pulse (We set the parameters to 20 V, 50 ms length, 50 ms interval, five-times).

12. Deliver 10 µg of CD-vector mixture (ratio of each vectors; hOCT4-CD:hSK-CD:hUL-CD = 1.5:1:1) into 2,000,000 cells using a NEPA21 Super electroporator (*see Note 3*).
13. Add the electroporated cells into 6 mL hFM, and mix well by pipetting slowly.
14. Seed one third of the electroporated cells onto one well of a Geltrex-coated 6-well plate.
15. Incubate the cells in a humidified 37 °C incubator.

3.2.2 hiNPC Reprogramming

1. (Day 1), Aspirate the medium and wash the cells once with equal volume of DPBS to remove the dead cells.
2. Add fresh hFM every other day thereafter.
3. (Day 5), Remove the medium and wash the cells once with DPBS.
4. (Day 6–24), Add 2 mL freshly prepared prewarmed hiNPC medium. Culture the cells for additional 15–19 days. Replace the spent medium with fresh prewarmed hiNPC medium every other day (*see Note 4*).

3.2.3 Selection and Isolation of the Successfully Reprogrammed Cells

1. Two to three weeks after electroporation, colonies would start to appear. When the colony is about 100 µm in width, they are ready for isolation.
2. Successfully reprogrammed hiNPC colonies have smooth edges and densely packed cells, which are morphologically distinguishable from the rest of the culture (Fig. 2).
3. Manually pick up the hiNPC colonies under a stereoscopic microscope installed inside a biosafety cabinet. In detail, center the hiNPC colony and draw a grid around it with a Precision-Glide needle. Using a 10-µL pipette tip, scrape off the edges of the marked colony and detach it from the culture dish.
4. Collect the colony into a 1.5 mL tube with medium and then centrifuge at $150 \times g$ for 5 min at room temperature. Remove the supernatant carefully.
5. Add 1 mL Accutase, then incubate at 37 °C for 5 min.
6. Briefly pipet the cells in the tube to mix and centrifuge at $150 \times g$ for 5 min at room temperature.
7. Transfer the dissociated cells onto one well of a Geltrex-coated 4-well dish. The passage number of the isolated colonies is zero (P0) (*see Note 5*).

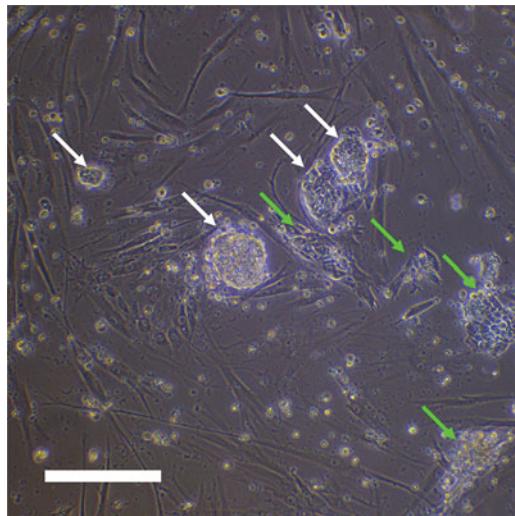


Fig. 2 Representative bright field image of directly reprogrammed colonies. White arrows indicate successfully reprogrammed hiNPC colonies. These colonies contain the densely packed cells and exhibit a characteristic dome-shaped morphology with smooth edges. Green arrows indicate patches of cell aggregates where the cell-fate conversion has been unsuccessful. Note the morphological differences readily distinguishable between these aggregates and the hiNPC colonies. Moreover, these cell aggregates do not express hNPC markers when tested. The cells with elongated morphology in the background are fibroblasts. The image is acquired on day 18 of reprogramming. Scale bar represents 100 μm

3.3 Removal of CD-Episomal Vectors and Copy Number Detection

3.3.1 Rapid Elimination of the Exogenous CD-Episomal Vectors

1. Culture the hiNPCs for additional 7–10 days with hiNPC medium containing 50 μM 5-FC.
2. Scrape off half of the cells in each well using a 10- μL pipette tip, then harvest and transfer the detached cells to a 1.5 mL tube. Proceed to Subheading 3.3.2 to use the harvested cells. The remaining cells in the culture dish will proceed to the next step.
3. Aspirate the used medium and wash the cells once with DPBS.
4. Add 300 μL Accutase solution and incubate the culture dishes at 37 °C for 5 min.
5. Collect and transfer the cell suspension into a 1.5 mL tube. Then pipet up and down to dissociate the cells into single cells.
6. Centrifuge at 150 $\times g$ for 5 min at room temperature.
7. Aspirate the supernatant carefully. Add 500 μL hiNPC medium containing 50 μM 5-FC. Pipet up and down to dissociate the cell pellets.
8. Seed the completely dissociated cells onto one well of a Geltrex-coated 4-well dish.

Table 1
Master mix preparation for vector copy number detection

Reagent	Volume per reaction (μ L)
iQ SYBR green super mix	35
DNA	7
D.W.	14

9. For next subculture, repeat **steps 3–7**. Seed half of the collected cells onto a Geltrex-coated 35 mm culture dish or appropriate amount suitable for your experimental objective. Use the remaining half of the collected cells in Subheading **3.3.2**.

3.3.2 Preparation of DNA Including Genomic and Exogenously Introduced DNA

1. Spin down the cells from the **step 2** or **9** in Subheading **3.3.1** at $150 \times g$ for 5 min at room temperature.
2. Remove the supernatant carefully and add 90 μ L DirectPCR Reagent as well as 10 μ L Proteinase K solution. Transfer the solution to a PCR tube.
3. Run the C1000 thermal cycler with the following parameters: 55 °C for 3 h, 85 °C for 30 min, and 12 °C for hold.
4. DNA samples can be stored at 4 °C prior to use.

3.3.3 Quantitative PCR for Copy Number Detection

1. Prepare master mix solution according to Table 1 including no template control, positive control (CD-vector transfected fibroblasts), standards for *EBNA1* and *FBXO15* (which should be run on every plate). For standard curve generation, a ten-fold serial dilution (from 10 ng to 10^{-5} ng) of pCXLE-hFbx15-cont2 plasmid is used. For example, a 96-well plate setup is described in Table 2.
2. Divide equal volume of the master mix solution into two 1.5 mL tubes. Add the *EBNA1* primer set into one tube and the *FBXO15* primer set into the other (7 μ L of 0.1 nM forward and reverse primer mix per tube).
3. Transfer 10 μ L of master mix solution with primer set into each well of the PCR plate as shown in Table 2.
4. Seal plate with optical film.
5. Briefly centrifuge the PCR plate.
6. Run the 7500 Fast real-time PCR system with method for 10 μ L volume.

Table 2
Example of reaction plate set up

	1	2	3	4	5	6	7	8	9	10	11	12
A	E1	E1	E1	F1	F1	F1	ES1	ES1	ES1	FS1	FS1	FS1
B	E2	E2	E2	F2	F2	F2	ES2	ES2	ES2	FS2	FS2	FS2
C	E3	E3	E3	F3	F3	F3	ES3	ES3	ES3	FS3	FS3	FS3
D	E4	E4	E4	F4	F4	F4	ES4	ES4	ES4	FS4	FS4	FS4
E	E5	E5	E5	F5	F5	F5	ES5	ES5	ES5	FS5	FS5	FS5
F	E6	E6	E6	F6	F6	F6	ES6	ES6	ES6	FS6	FS6	FS6
G	E7	E7	E7	F7	F7	F7	ES7	ES7	ES7	FS7	FS7	FS7
H	EPC	EPC	EPC	FPC	FPC	FPC	ENT	ENT	ENT	FNT	FNT	FNT

The number after the alphabet (E, F, ES, and FS) represents individual sample or serially diluted standard curve sample. E1 = sample 1 with *EBNA1* primer set; F1 = sample 1 with *FBXO15* primer set; ES1 = standard curve sample 1 with *EBNA1* primer set; FS1 = standard curve sample 1 with *FBXO15* primer set; EPC = positive control with *EBNA1* primer set; FPC = positive control with *FBXO15* primer set; ENT = no template control with *EBNA1* primer set; FNT = no template control with *FBXO15* primer set

3.3.4 Copy Number Detection

1. The copy numbers in each sample is calculated by using standard curves as follows.
2. The average molecular weight of a DNA base pair is 650 Da. The pCXLE-hFbx15-cont2 plasmid is 10,180 base pairs in length, with a molecular weight (MW) of 6,617,000.
3. The weight of one plasmid is MW/Avogadro constant. Thus, weight of one pCXLE-hFbx15-cont2 plasmid is:

$$6.617 \times 10^6 / 6.022 \times 10^{23} \text{ g} = (6.617 \times 10^6 / 6.022 \times 10^{23}) \times 10^9 \text{ ng}$$

4. Therefore, standard 1 (contains 10 ng of control plasmids) contains:

$$10 / \{(6.617 \times 10^6 / 6.022 \times 10^{23}) \times 10^9\} \approx 9.1008 \times 10^8 \text{ copies}$$

5. Generate standard curves for *EBNA1* and *FBXO15* (Fig. 3).
6. The Ct value of *FBXO15* and *EBNA1* are used to determine the cell number and CD-vector copy number, respectively. The number of copies per cell are calculated by dividing the total CD-vector copy numbers by the cell numbers.

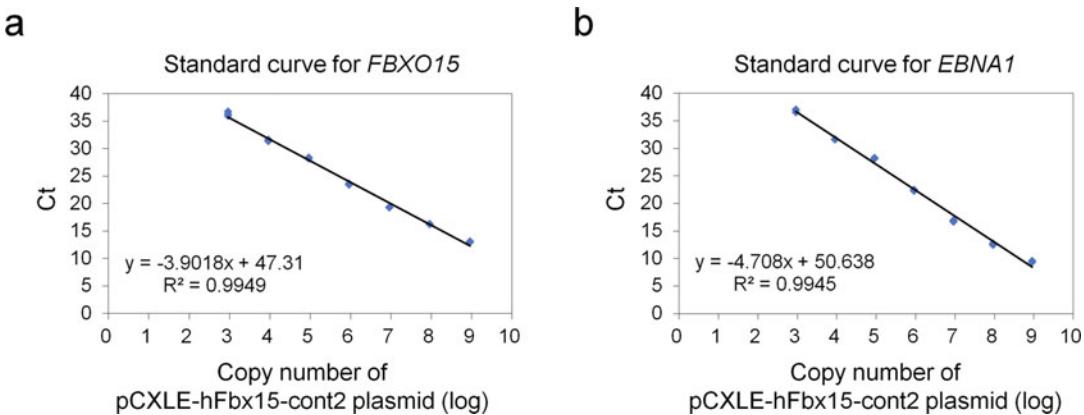


Fig. 3 Examples of standard curves for calculating the vector copy number per cell. **(a)** Standard curve for *FBXO15* to count cell number. **(b)** Standard curve for *EBNA1* to calculate vector copy number. The details of the procedure are described in the Subheading 3.3. *FBXO15* and *EBNA1* are used to determine the cell number and the copy number of the CD-episomal vectors, respectively. The copy number of episomal vectors is divided by the total cell counts to obtain the copy number per cell

3.4 Characterization of the hiNPCs

3.4.1 Immuno-cytochemistry

1. Remove the culture medium and wash the cells one time with DPBS.
2. Add 300 μ L fixative solution for each well of a 24-well culture dish and incubate for 10 min at room temperature, followed by three washes with DPBS.
3. Add P&B solution and incubate the cells for 1 h at room temperature.
4. Make primary antibody solution during the process of step 3. Add appropriate amount of antibody into 1% BSA solution.
5. Remove the P&B solution and add primary antibody solution. Incubate the samples overnight at 4 °C.
6. Wash three times with 500 μ L of 0.1% BSA solution each time for a total of 1 h.
7. Make the secondary antibody solution during the process of step 6. Dilution ratio is described in Subheading 2.4.1. Add appropriate amount of antibody into 0.1% BSA solution.
8. Remove 0.1% BSA solution and add the secondary antibody solution. All samples are then incubated for 1 h at room temperature.
9. Wash three times with 500 μ L of 0.1% BSA solution each time for a total of 1 h.
10. Make the working Hoechst33342 solution at a final concentration of 0.2 μ g/mL Hoechst33342 in 0.1% BSA solution.

11. Add 500 μ L of the working Hoechst33342 solution and incubate for 15 min at room temperature.
12. Examine the staining results and capture the images using a fluorescence microscope (*see Note 6*).

3.4.2 Coating the Plate for hiNPC Differentiation (See Note 7)

1. Add 300 μ L poly-L-ornithine solution into each well of a 24-well plate. Coat the plate by incubation at 37 °C for at least 1 h.
2. Remove the poly-L-ornithine solution and wash the coated wells twice with 600 μ L DPBS each time.
3. Add 300 μ L laminin solution to each well of the poly-L-ornithine-coated 24-well plate. Conduct the second coating of the plate by incubation at 37 °C for at least 1 h.

3.4.3 hiNPC Differentiation (See Note 7)

1. (Day 0), Remove the culture medium, then wash hiNPCs once with DPBS. Aspirate the used DPBS.
2. Add 1 mL Accutase for one 35 mm culture dish and incubate the plate at 37 °C for 5 min.
3. Gently pipet the cells to generate a single-cell suspension and collect the cell suspension into a 15 mL tube.
4. Centrifuge at $150 \times g$ for 5 min at room temperature.
5. Remove the supernatant carefully and add 5 mL fresh hiNPC medium.
6. Thoroughly resuspend the cells by pipetting up and down using a 1-mL pipette without producing air bubbles.
7. Count the live cells using trypan blue staining and a hemacytometer. Alternatively, one can use automated systems like Countess (Thermo Fischer) or NC-200 (Chemometec).
8. Seed the cells at the density of 1.5×10^5 cells/cm² onto one well of a 24-well plate doubly coated with poly-L-ornithine and laminin.
9. (Day 1) Remove the used medium and wash one time with DPBS. Aspirate the DPBS.
10. Add 500 μ L ND medium into each well.
11. Culture the cells for 21 days. Half the volume of total medium in each well is replaced every 3 day.
12. (Day 14, the day of analysis), Perform the ICC for the neuronal and glial markers. Representative photomicrographs of the immunostained cells are provided in Fig. 4 (*see Note 8*).

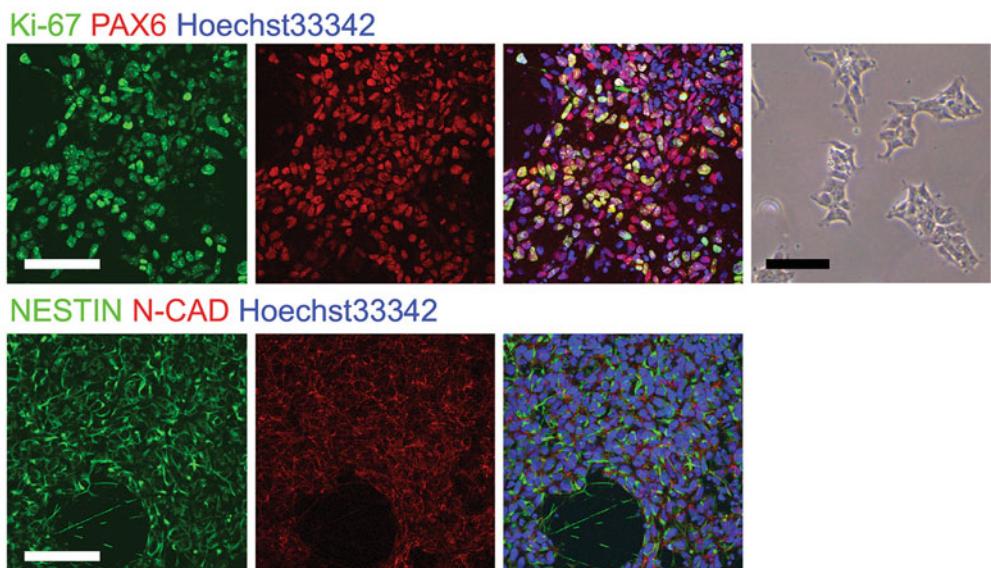
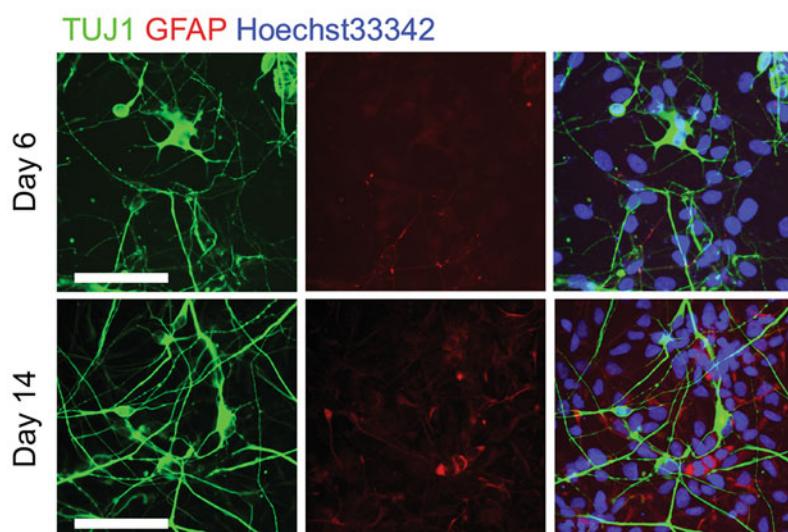
a**b**

Fig. 4 Characterization of hiNPCs. **(a)** Top Panel: Representative bright field micrographs of a low-density adherent culture of hiNPCs is shown in the far-right panel. Note the typical neuroepithelial morphology of the neural progenitor cells. The remaining images are from cells that were fixed and immunostained for NPC markers. Cells display double immunopositivity to PAX6 (Red) and proliferation marker Ki67 (Green). Nuclei stained with Hoechst33342 are shown in blue. Note, nearly 100% of the cells are proliferative at this progenitor stage, as indicated by the co-labeled cells in the merged image. Bottom panel: Cells were also double immunopositive for N-CAD (CDH2) (Red) and NESTIN (Green). Nuclei stained with Hoechst33342 are shown in blue. **(b)** Differentiation of hiNPCs to neurons and glia. Representative immunohistograms of cultures when examined on day 6 and day 14 of differentiation. The majority of the cells displayed immunoreactivity to the neuronal marker TUJ1 (Green) on both days analyzed. These cells also gained more complex neuronal morphology as differentiation progressed. A proportion of the cells also expressed the astroglial marker GFAP (red). The right panel is a merge of images from the same field captured under the green, red, and blue channel. Nuclei stained with Hoechst33342 are shown in blue. Scale bars represent 50 μ m in **(a)** and 25 μ m in **(b)**.

4 Notes

1. Vortexing may cause fragmentation of laminin. Gently pipet using a 1-mL pipette to mix.
2. We recommend that you optimize the seeding density for each cell type used. Previously, we subcultured the CRL-2097 fibroblasts (ATCC) at 1:4 ratio every 7 days when they reach a confluence of ~90% [12].
3. Since fibroblasts from different donors may differ in their transfection efficiency, further optimization of the electroporation conditions may be required for your cells.
4. On the day after the medium change, the culture medium may look pink or yellow depending on your cell types. Yellow coloration indicates acidity resulting from rapid cell growth. If this is the case, it is recommended to replace the medium every day.
5. We typically obtained 12–20 hiNPC colonies per electroporation of 2,000,000 cells. This roughly translates to hiNPC reprogramming efficiency of ~0.001%.
6. We recommend characterization of hiNPCs for genomic stability by karyotype analysis and/or whole genome sequencing. The donor identity is confirmed by short tandem repeat (STR) analyses.
7. The hiNPC differentiation method provided here mostly produce cortical glutamatergic neurons, and a minor population of astroglial cells. Other robust protocols may be employed for directed differentiation of hiNPCs to generate a different type of neurons [13], astroglia [14], or oligodendroglia [15].
8. Additional images from the immunocytochemical characterization of differentiated cells can be found in our previous publication [8].

Acknowledgments

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Chapter 6

Generation of Human Neurons by microRNA-Mediated Direct Conversion of Dermal Fibroblasts

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Abstract

MicroRNAs (miRNAs), miR-9/9^{*}, and miR-124 (miR-9/9^{*}-124) display fate-reprogramming activities when ectopically expressed in human fibroblasts by erasing the fibroblast identity and evoking a pan-neuronal state. In contrast to induced pluripotent stem cell-derived neurons, miRNA-induced neurons (miNs) retain the biological age of the starting fibroblasts through direct fate conversion and thus provide a human neuron-based platform to study cellular properties inherent in aged neurons and model adult-onset neurodegenerative disorders using patient-derived cells. Furthermore, expression of neuronal subtype-specific transcription factors in conjunction with miR-9/9^{*}-124 guides the miNs to distinct neuronal fates, a feature critical for modeling disorders that affect specific neuronal subtypes. Here, we describe the miR-9/9^{*}-124-based neuronal reprogramming protocols for the generation of several disease-relevant neuronal subtypes: striatal medium spiny neurons, cortical neurons, and spinal cord motor neurons.

Key words Neuronal reprogramming, miRNA-mediated direct conversion, Aging, Neuronal cell-fate, Human neuron, miN, Medium spiny neuron, Motor neuron, Cortical neuron, Neurogenesis

1 Introduction

During mammalian neural development, microRNAs-9/9^{*} and -124 (miR-9/9^{*}-124) are upregulated at the onset of neurogenesis and function as a molecular switch to promote neuronal differentiation. Some of these switching mechanisms include the specification of neuron-specific subunit composition of BAF chromatin remodeling complexes, turning off anti-neurogenic transcription factors, and switching to neuron-specific alternative splicing [1–4]. Interestingly, many of the direct targets of miR-9/9^{*}-124 are genes typically expressed in most non-neural somatic cell types. Further, when ectopically expressed in non-neural cell

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types, these miRNAs exhibit neurogenic activities such as the direct fate conversion of skin fibroblasts to neurons [5, 6]. Importantly, miR-9/9^{*}-124 can reprogram human fibroblasts from donors across the life spectrum while retaining the epigenetic age information stored in fibroblasts [7], thereby allowing generation of human neurons that reflect all ages [8]. Studies from human adult fibroblast conversion identified that miR-9/9^{*}-124 drive fate conversion via reconstruction of the chromatin landscape [9] in part through repression of the neuronal RE1-silencing transcription factor (REST) [10] and induction of neuronal BAF subunit switching [2]. As such, the switch to the neuronal BAF complex drives chromatin remodeling that activates neuronal genes, leaving cells in the miRNA-induced neuronal state, poised to respond to transcription factors that promote neuronal maturity and subtype specificity [9]. The concordant expression of neuronal transcription factors with miR-9/9^{*}-124 generates subtype-specific neuronal programs and increases neuronal maturity. Currently, the miRNA-based reprogramming protocols allow the generation of the following neuronal subtypes: cortical neurons (CNs), striatal medium spiny neurons (MSNs), and spinal motor neurons (MNs). These neuronal subtypes are afflicted in neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, and Amyotrophic lateral sclerosis, and thus generation of neurons with miR-9/9^{*}-124 in combination with neuron-specification transcription factors opens the door to the investigation of subtype-specific disease pathology.

Aging is the greatest risk factor in most forms of neurodegenerative diseases and thus age needs to be recapitulated in reprogrammed, patient-derived cells for modeling late-onset disorders. This requirement poses an experimental challenge for human neurons differentiated from induced pluripotent stem cells since the pluripotency induction reverts the reprogramming cells to an embryonic state, which produces neurons that mimic fetal stages and hinders the studies of cellular properties intrinsic in aged neurons [7, 11, 12]. Direct neuronal conversion of human fibroblasts bypasses the stem cell stages and retains age-associated signatures such as transcriptomic changes [13], miRNA expression, and epigenetic age [8]. The significance of age in modeling late-onset disorders has been recently demonstrated. For instance, MSNs, the primary cell type affected in Huntington's disease (HD), when reprogrammed from adult-onset HD patient fibroblasts exhibit hallmarks of HD pathology, including aggregation of the endogenous mutant HTT protein, increased DNA damage, and spontaneous neuronal death. Importantly, these phenotypes were not observed in neurons differentiated through an iPS or embryonic-like state. Further, the degree of pathogenicity observed in these phenotypes was sensitive to the cellular age when the fibroblasts were sampled [14]. Thus, the success of modeling late-onset neurodegenerative diseases relies on multiple,

equally important factors: (a) efficiency of neuronal reprogramming of fibroblasts from adult individuals to functionally mature neurons, (b) control for neuronal subtype specificity, and (c) cellular age manifested in converted human neurons.

An important feature in any neuronal reprogramming is identifying and defining neuronal maturity. Staining with markers like Beta-Tubulin III (TUBB3) demonstrates neuronal morphologies but does not define neuronal cell fate. The expression of specific neuronal markers indicates that reprogrammed cells have reached a pan-neuronal fate (e.g., MAP2, NEFL), but does not identify fully differentiated neurons. Functional characterization by whole-cell recording is a mandatory measure for defining neuronal fate. Yet, even induced action potential firing alone can be biased to cells picked for recording. Our laboratory recently started employing a systematic transcriptomic feature in which the neuronal identity is also defined through expression of long genes (>100 kb long), a feature unique to fully differentiated, functional neuronal populations [15]. We routinely find that the functional maturation of converted neurons as measured through electrophysiology and long gene expression, peaks when miR-9/9^{*}-124 synergize with subtype-specific transcription factors that activate mature neuronal gene networks [9]. These fate indicators (presence of neuronal markers, pan-neuronal and long gene expression signature, and electrophysiological properties) should be collectively assessed to examine the adoption of neuronal identity through direct reprogramming.

In miRNA-based neuronal reprogramming of human fibroblasts, reprogramming with only miR-9/9^{*}-124 first is often a good starting point to monitor the process of neuronal conversion. Although reprogramming with miR-9/9^{*}-124 alone may not generate fully mature subtype-specific neurons, it provides a foundation for monitoring morphological transition of fibroblasts to neurons. In this chapter, we first describe the protocol to generate miRNA-induced neurons (miNs) from adult human fibroblasts using miR-9/9^{*}-124 (Fig. 1) as reprogramming with miRNAs alone is critical to become familiar with how reprogramming cells behave during miRNA-mediated neuronal reprogramming. We then describe how to generate MSNs, MNs, and CNs using miR-9/9^{*}-124 together with subtype-defining transcription factors (CTIP2, DLX1, DLX2, and MYT1L; LHX3 and ISL1; and NEUROD2 and MYT1L, respectively) (Fig. 2) [5, 9, 14, 16, 17].

2 Materials

2.1 Cells

1. Lenti-X 293T cell line (293LE).
2. Primary human fibroblasts.

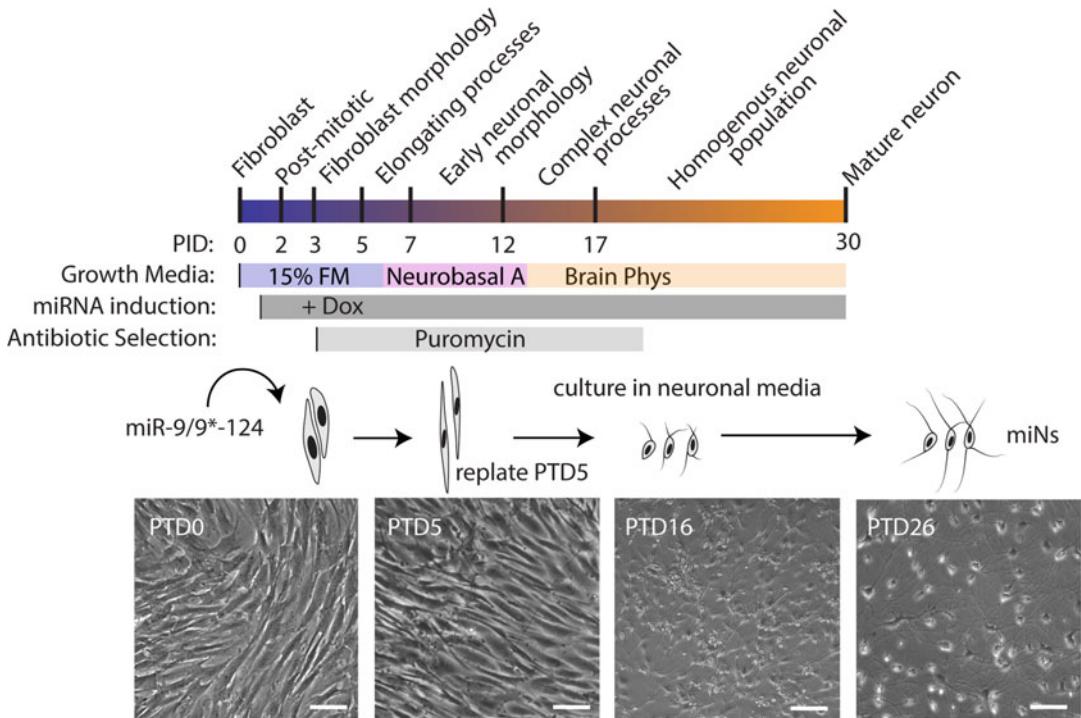


Fig. 1 miR-9/9^{*}-124-mediated direct conversion of human fibroblasts to neurons. Reprogramming timeline indicating distinguishable phenotypes throughout conversion. Phase images of miR-9/9^{*}-124-derived cells at PTD0, 5, 16, and 26. Fibroblasts on PTD0 are fully confluent, become post-mitotic by PTD2–3, and typically elongate by PTD5 (the phase image of PTD5 cells was taken before replating). Note that PTD5 cells have kept the high cell density in the presence of antibiotics due to efficient lentiviral transduction. Around or before PTD14, cells are usually fully neuronal. Cells begin to mature around PTD26, with full maturity reached around PTD35. Scale bars are 100 μ M

2.2 Reagents for Transfection/Transduction

1. rtTA-N144 (hygromycin mammalian resistance; Addgene, 66810).
2. pTight-9-124-BclxL (miN reprogramming; Addgene, 60857).
3. Opti-MEM reduced-serum medium.
4. Polyethylenimine “Max”, MW 40,000 Da (PEI).
5. Hexadimethrine bromide (Polybrene).
6. Second generation lentiviral packaging vectors, psPAX2 (packaging vector) and pMD2.G (envelope vector).
7. pmCTIP2-N106 (MSN reprogramming; Addgene, 66808).
8. phDLX1-N174 (MSN reprogramming; Addgene, 60859).
9. phDLX2-N174 (MSN reprogramming; Addgene, 60860).
10. phMYT1L-N174 (MSN and CN reprogramming; Addgene, 66809).
11. phNEUROD2-N174 (CN reprogramming; Addgene, 31822).

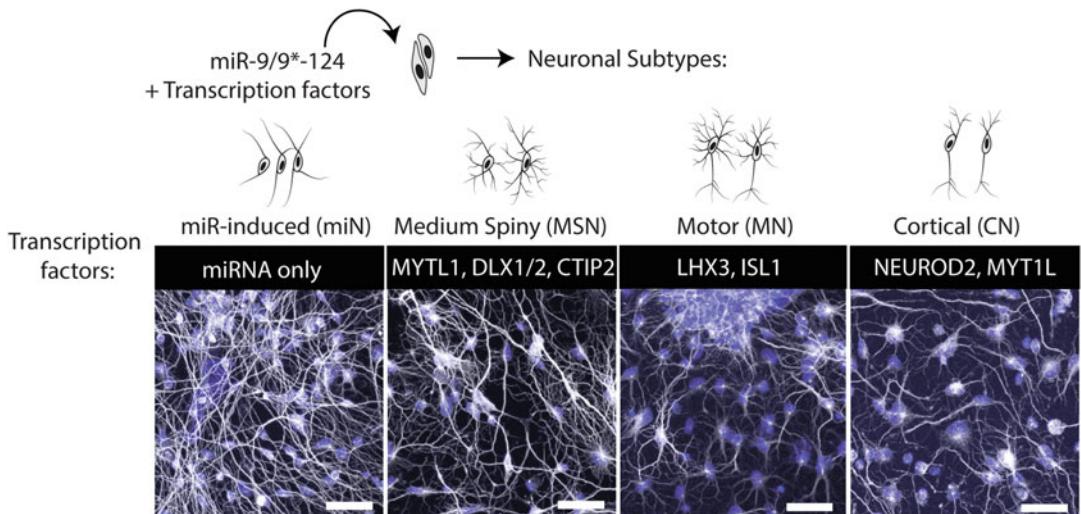


Fig. 2 Addition of neuronal-specific transcription factors to miR-9/9^{*}-124-mediated direct conversion allows generation of neuron-specific subtypes. Morphological differences between mature neuronal subtypes (PTD35) are visualized by staining for TUBB3 (gray) and DAPI (blue). Scale bars are 20 μM

12. phISL1-N174 (MN reprogramming).
13. phLHX3-N174 (MN reprogramming).

2.3 Reagents for Fibroblast and 293LE Cell Culture

1. DMEM (Dulbecco's Modified Eagle Medium), high glucose, no glutamine.
2. FBS, qualified, US Department of Agriculture (USDA)-approved regions.
3. Minimum essential medium (MEM) non-essential amino acid solution (NEAA, 100 \times).
4. Sodium pyruvate (NaPyr, 100 mM).
5. GlutaMAX supplement.
6. Penicillin-streptomycin (10,000 U/mL Penicillin and 10,000 $\mu\text{g}/\text{mL}$ Streptomycin).
7. HEPES (1 M) buffer solution.
8. β -Mercaptoethanol.
9. Trypsin-EDTA (0.05%, wt/vol), phenol red.
10. Trypsin-EDTA (0.25%, wt/vol), phenol red.
11. Dulbecco's PBS (DPBS), no calcium, no magnesium.
12. High-speed/ultracentrifuge capable of centrifugation at 70,000 $\times \mathcal{g}$ and 4 °C (such as Beckman Coulter Avanti JXR-30 Centrifuge + Beckman JA-25.50 rotor).

2.4 Reagents for Reprogramming Neurons

1. NeurobasalA (Gibco).
2. B-27 plus supplement (Gibco).
3. GlutaMAX supplement (Gibco).

4. BrainPhys (Stemcell).
5. NeuroCult SMI neuronal supplement (Stemcell).
6. N2 supplement-A (Stemcell).
7. Dibutyryl-cAMP sodium salt (dB-cAMP).
8. Valproic acid (VPA), sodium salt.
9. Recombinant human brain-derived neurotrophic factor (BDNF).
10. Recombinant human neurotrophin-3 (NT-3).
11. Retinoic acid (RA).
12. RevitaCell Supplement (RVC).
13. Doxycycline hyclate (DOX).
14. Puromycin dihydrochloride.
15. Blasticidin S HCl.
16. Geneticin selective antibiotic (G418 sulfate).
17. Centrifuge and swing bucket rotor for spinfection (such as Eppendorf Centrifuge 5810 R 15 amp version and swing rotor IL 109).

**2.5 Reagents
for Preparation
of Glass Coverslips,
and Replating of Cells
on Coverslips**

1. Alconox detergent packets.
2. Autoclaved deionized water.
3. Ethanol, 95% (vol/vol).
4. Nitric acid, 70% (wt/wt).
5. Poly-L-ornithine solution, 0.01% (Sigma-Aldrich).
6. Autoclaved Milli-Q water.
7. Laminin, aqueous solution, 1 mg/mL (Sigma-Aldrich).
8. Fibronectin, powder (Sigma-Aldrich).
9. Corning™ Primaria™ Tissue Culture Dishes.
10. PYREX® 150 × 20 mm Petri Dish with Cover.
11. Tissue culture plates (6-, 12-, and 24-well plates).
12. Glass coverslips (25, 18, and 12 mm, for replating in 6-, 12-, and 24-well plates, respectively).

**2.6 Fibroblast
Medium (FM) (with
~10% or ~15% FBS,
Named as FM10
and FM15,
Respectively)**

DMEM supplemented with ~10 or 15% FBS, 1 mM NaPyr, 1 × NEAA, 1% GlutaMAX, 100 U/mL Penicillin and 100 µg/mL Streptomycin, 0.1% β-mercaptoethanol). Into the bottle containing 500 mL DMEM basal media, add 50 or 75 mL of FBS (FM10 and FM15, respectively). Then, add 5 mL MEM NEAA solution, 5 mL NaPyr, 5 mL GlutaMAX, 5 mL penicillin-streptomycin, and 0.5 mL of β-mercaptoethanol. Invert several times to mix and store the media at 4 °C for up to 1 month.

2.7 Polyethylenimine (PEI) Solution

1 mg/mL PEI in water, pH 7.0. First, make a 2 mg/mL PEI solution in autoclaved Milli-Q water. Adjust the pH to 7.0 using NaOH. Then, adjust the volume with Milli-Q water to a working PEI concentration of 1 mg/mL. Sterilize the solution through a 0.22-µm mixed cellulose ester (MCE) filter, aliquot in 1 mL volumes, and store them at –80 °C for up to 1 year. PEI solution is only stable at 4 °C for 1 week.

2.8 Polybrene (PB) Solution

8 mg/mL PB in water. Prepare an 8 mg/mL stock solution of PB in autoclaved Milli-Q water and sterilize the solution by using a 0.22-µm MCE filter. Store 500-µL aliquots at –20 °C for up to 2 years. Aliquots can be thawed and stored at 4 °C for a month. The thawed aliquots cannot be re-frozen.

2.9 Dibutyl-cAMP Stock Solution

200 mM dB-cAMP in water. Add 1 g of dB-cAMP powder into 10.2 mL autoclaved Milli-Q water to make a 200 mM stock solution and sterilize it by using a 0.22-µm MCE filter. Store aliquots at –20 °C for up to 6 months. Aliquots can be thawed and stored at 4 °C for a month. Thawed aliquots cannot be re-frozen.

2.10 Valproic Acid (VPA) Stock Solution

1 M VPA in water. Add 1.66 g of VPA sodium salt into 10 mL of autoclaved Milli-Q water and mix to make a 1 M stock solution and sterilize it with an 0.22-µm MCE filter. Store aliquots at –20 °C for up to 3 months. The thawed aliquots can be stored at 4 °C for 2 weeks. The thawed aliquots cannot be re-frozen.

2.11 Human BDNF Stock Solution

1 mg/mL BDNF in water. Before opening, centrifuge the product vial at 2000 × g for 30 s at 25 °C. Add enough Milli-Q water into the vial to make a concentration of 1 mg/mL (2000×). Do not vortex. Further dilute the solution in PBS + 0.1% (wt/vol) BSA to a 20 µg/mL working concentration for extended storage. Store the working aliquots at –20 °C for 12 months. Aliquots can be thawed and stored at 4 °C for 2 weeks. Thawed aliquots cannot be re-frozen.

2.12 Human NT-3 Stock Solution

1 mg/mL NT-3. Before opening, centrifuge the product vial at 2000 × g for 30 s at 25 °C. Add enough Milli-Q water into the product vial to make a concentration of 1 mg/mL (2000×). Do not vortex. Further dilute the solution in PBS + 0.1% (wt/vol) BSA to a 20 µg/mL working concentration for extended storage. Store the working aliquots at –20 °C for 3 months. Aliquots can be thawed and stored at 4 °C for 2 weeks. The thawed aliquots cannot be re-frozen.

2.13 Retinoic Acid (RA) Solution

2 mM RA in DMSO. Add 6 mg of RA powder into 10 mL of DMSO and mix (vortexing may be needed) to make a 2 mM stock solution (2000×) and sterilize the solution with a 0.22-µm nylon

filter. Store aliquots at -20°C for up to 1 year. Aliquots can be thawed and stored at 4°C for 2 weeks. The thawed aliquots cannot be re-frozen. Protect from light.

2.14 Geneticin Selection (G418) Solution

50 mg/mL G418. Mix G418 powder in Milli-Q water to a concentration of 50 mg/mL and sterilize it through a 0.2- μm MCE filter. Store the aliquots at -20°C and thaw at 4°C before use.

2.15 Doxycycline (DOX) Solution

1 mg/mL DOX. Add 40 mg of DOX powder into 40 mL of autoclaved Milli-Q water and mix to make a 1 mg/mL stock solution ($1000\times$). Sterilize the solution with a 0.22- μm MCE filter and store the aliquots at -20°C for up to 6 months. The aliquots can be thawed and stored at 4°C for 1 week. Do not re-freeze the thawed aliquots. Protect from light.

2.16 Fibronectin Solution

200 $\mu\text{g}/\text{mL}$ fibronectin in water. Reconstitute the fibronectin powder in Milli-Q water to a concentration of 200 $\mu\text{g}/\text{mL}$ and sterilize it through a 0.2- μm MCE filter. Aliquot in 500 μL aliquots and store them at -20°C . Do not re-freeze the thawed aliquots.

2.17 Reprogramming Medium I

NeurobasalA media containing 1 \times B-27 plus supplement and 1 \times GlutaMAX. Into a bottle containing 500 mL of NeurobasalA media, add 10 mL of the 50 \times B-27 plus supplement and 5 mL of the 100 \times GlutaMAX. Reprogramming medium I should be stored at 4°C and used within 1 month. Protect from light.

2.18 Reprogramming Medium II

BrainPhys containing 1 \times NeuroCult SM1 neuronal supplement and 1 \times N2 supplement-A. Into a bottle containing a 500 mL of BrainPhys, add 10 mL of 50 \times NeuroCult SM1 neuronal supplement, and 5 mL of 100 \times N2 supplement-A. Reprogramming medium II should be stored at 4°C and used within 1 month. Protect from light.

2.19 Alconox Detergent Solution

Alconox detergent water. Add one 0.5-ounce (~14.2 g) package of Alconox Detergent powder to a glass bottle containing 2 L of deionized water. Place the bottle in a 37°C water bath for 30 min to 1 h or until all of the Alconox powder is dissolved.

3 Methods

3.1 Lentivirus Packaging and Concentration by High-Speed Centrifugation

- On day 1, plate 7–8 million 293LE cells per 10-cm culture dish in FM10 medium.
- On day 2, the cells in the culture dish should be 80% confluent (Fig. 3, see Note 1). Make one transfection mastermix for each 10-cm plate in a 1.5 mL centrifuge tube by adding the following reagents in the order listed: 1.5 μg of pMD2.G, 4.5 μg of

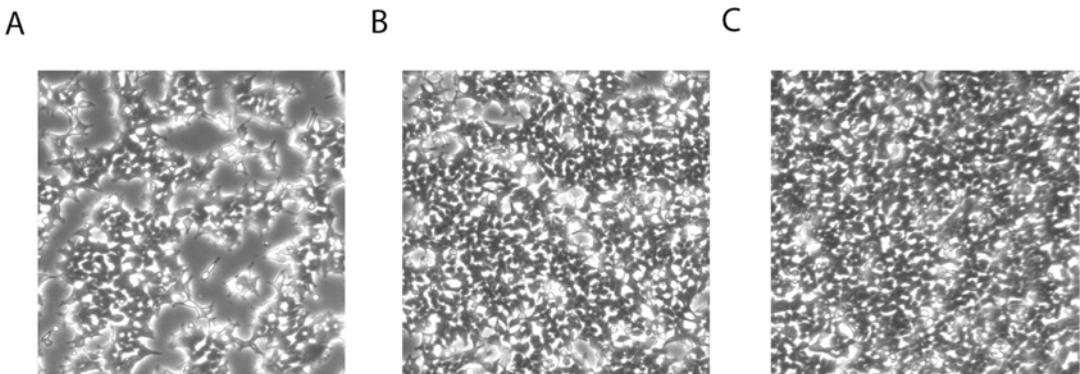


Fig. 3 293LE cells that are 80–90% confluent on the day of transfection produce optimal titers of lentivirus for our reprogramming conditions. 293LE cells are shown at three different densities: underconfluent (**a**), 80–90% confluent (correct confluence) (**b**), and overconfluent (**c**). Scale bars are 100 μM

psPAX2, 6 μg of lentiviral plasmid (e.g., pT-9124-BclxL), 600 μL of Opti-MEM, 48 μL of PEI solution. Be sure to add the PEI last (*see Note 2*).

3. Invert the microcentrifuge tubes 2–3 times and then centrifuge briefly using minicentrifuge.
4. Incubate the transfection mix at room temperature for 20 min. Incubation of the transfection mix should not go over 20 min as longer incubation may decrease the transfection efficiency.
5. Without mixing, use a p1000 pipette to add the transfection mix dropwise to different areas of the 10-cm dish.
6. Gently mix by moving the plate sideways in all directions and incubate it in the 37 °C incubator overnight for up to 16 h. It is recommended that the transfection does not last for more than 16 h.
7. The next day (day 3) warm FM10 to 37 °C. Carefully aspirate the transfection media from the 10-cm dishes and add 10 mL of prewarmed FM10 along the wall of the dish so as not to dislodge the cells (*see Note 3*). Return the plates to the incubator and incubate for 48 h.
8. On day 5, carefully collect the virus supernatant from each 10-cm dish into a 15 mL conical tube. If you have multiple 10-cm plates containing the same virus, the virus-containing media can be combined into a 50 mL conical tube.
9. Centrifuge the virus supernatant at $1200 \times g$ for 5 min at 4 °C to remove any 293LE cell debris.
10. Filter the supernatant using a 0.45- μm polyethersulfone (PES) filter and collect each virus into a 50 mL centrifuge tube that is appropriate for your labs ultra-, or high-speed centrifuge and a 2 h, 4 °C spin at $70,000 \times g$.

11. Centrifuge the lentiviral supernatant at $70,000 \times g$ for 2 h at 4 °C using a high-speed or ultracentrifuge. After centrifugation, a transparent brown pellet can be seen at the bottom of the tube (*see Notes 4–6*).
12. Carefully aspirate the supernatant without disturbing the pellet.
13. Resuspend the pellet in 1 mL of cold DPBS per 10-cm dish of virus. To facilitate resuspension with a pipette, you may leave the pellet soaking in DPBS on ice or in a cold room for 2 h or overnight. This soaking step is not required, however doing so is useful if you have a lot of viruses as it will make the pellets easier to resuspend.
14. Aliquot the concentrated lentiviruses into 1.5-mL microcentrifuge tubes with 500 µL per tube and store them at –80 °C. Frozen lentiviruses can be stored at –80 °C for up to 1 year.

3.2 Coverslip Preparation

1. Place enough glass coverslips (~100 12-mm coverslips) to evenly cover the bottom of a 150-mm Pyrex glass dish without too much overlap.
2. Add enough Alconox detergent solution (prepared in Subheading 2.19, ~30 mL) to submerge all the coverslips and place the Pyrex dishes onto an orbital shaker set to shake slowly (about 150 rpm). Wash the coverslips while shaking at least 2 h or overnight.
3. Remove the alconox detergent solution and add an equal volume of autoclaved deionized water. Place the washing dish back on the orbital shaker and shake for 30 min. Repeat for a total of six washes with autoclaved deionized water.
4. Aspirate water from the coverslips and then add an equal volume of 95% (vol/vol) ethanol. Place the dishes back on the orbital shaker and slowly shake for 2 h at 150 rpm. After 2 h, remove the ethanol with a 25 mL serological pipette and dry the coverslips completely. Ethanol may get trapped between the coverslips and the dish. In addition, if the coverslips stack, ethanol may get trapped between the coverslips. Remove any residual ethanol and air-dry the coverslips. Residual ethanol left on the coverslips will react with nitric acid in the next step to produce dangerous and toxic nitrogen oxide gases.
5. Add 25 mL of 70% (wt/wt) nitric acid into the dish containing the coverslips in a fume hood (*see Note 7*). Place the dishes on an orbital shaker in a fume hood and shake slowly at about 150 rpm overnight (12–16 h).
6. Use a 25-mL serological pipette to remove the nitric acid from the dish and place it into a labeled waste container for chemical

disposal. Add 25–30 mL of autoclaved deionized water and wash the coverslips at 150 rpm on an orbital shaker for 30 min. Repeat for a total of eight washes.

7. After the last water wash, completely aspirate the water off the coverslips. It is essential to aspirate all of the water (rather than air-dry) at this step. Water may get trapped between the coverslips and the glass dish or between coverslips that have stacked. Failure to aspirate the water will result in the coverslips sticking together when dried.
8. Leave the dishes containing dried coverslips inside the tissue culture hood with UV light on overnight (at least 10 h). Remove the lids from the dishes but keep the lids inside the hood to be sterilized as well. Pretreated, sterilized coverslips may be prepared in advance and stored indefinitely in a dry, sterile container at room temperature until use.

3.3 miN Reprogramming

Ectopic expression of miR-9/9^{*}-124 in human fibroblasts induces neurogenic plasticity by triggering an extensive reconfiguration of the chromatin accessibility landscape that can be further guided by transcription factors to specific neuronal subtypes [9]. Thus, it will be critical to master the reprogramming process with miRNAs only before proceeding with subtype-specific neuronal reprogramming. The protocols below describe the basic reprogramming procedures with miRNAs only.

1. On post-transduction day-1 (PTD-1), replate confluent human fibroblasts into a fresh plate to be transduced. For a 6-well plate, use 0.3–0.4 million cells per well. If using a 12-well plate, scale the per-well number of cells by $\frac{1}{2}$ (*see Notes 8 and 9*).
2. Transduce the cells on the next day (PTD0) (*see Note 10*). First check the wells for confluence (Fig. 1, PTD0). If the cells are confluent, immediately proceed to **step 3**.
3. Thaw viruses on ice and spin for 5 min at 4 °C, 5000 $\times \text{g}$ to remove any debris.
4. Make the viral transduction mastermix. First warm FM15 to 37 °C. For a full 6-well plate, add 504 µL pTight-9124-BclxL and 504 µL of rtTA to a 50-mL conical tube. Then, add 24 mL of prewarmed FM15 medium followed by 24 µL of Polybrene (8 mg/mL or 1000 \times stock) to a conical tube. Add 4 mL of transduction mastermix per well.
5. Using a swing-bucket rotor, spinfect plates for 30 min at 37 °C and 1000 $\times \text{g}$ and incubate plates in the incubator overnight.
6. On PTD1, warm FM15 media to 37 °C and add DOX (1000 \times from 1 mg/mL stock) into the prewarmed FM15 to a final concentration of 1 µg/mL. Aspirate the viral media from the

cells. Wash the cells with sterile DPBS. After removing the DPBS, feed the cells with the prewarmed FM15 + 1 µg/mL DOX. Return the cells to the incubator. When adding either DPBS or media, add to the side of the well rather than directly on top of the cells.

7. On PTD3, aspirate the media and add fresh FM15 + 1 µg/mL DOX + 3 µg/mL Puromycin (final concentrations).
8. On PTD3–PTD5, we coat the coverslips with Poly-L-ornithine, fibronectin, and laminin to create an extracellular-like matrix for the cells to adhere to. To begin coating, on PTD3 move a single sterile coverslip to each well (from **step 8** of Subheading 3.2) of a 6-, 12-, or 24-well plates as needed (*see Note 11*). We use different sized coverslips for 6-, 12-, and 24-well plates (25-, 18-, or 12-mm). For a 6-well or 25-mm coverslip, add 160 µL of 0.01% Poly-L-ornithine to the center of the coverslip. Use caution to keep it from slipping off the glass. If using 12- or 24-well plates, scale the volume by $\frac{1}{2}$ and $\frac{1}{4}$, respectively, to account for the difference in area between coverslips. Incubate the coverslips with Poly-L-ornithine at 37 °C overnight.
9. On PTD4, wash the coverslips three times with autoclaved water. Directly to the coverslip, add sterile DPBS containing 2 µg/mL Fibronectin (200 µg/mL stock is 100 \times) and 5 µg/mL Laminin (1 mg/mL stock is 200 \times). Use the same volumes as with Poly-L-ornithine and incubate the plates in the 37 °C incubator overnight. Use caution to keep the coating solution from slipping off the glass while transferring the plate back to the incubator.
10. On PTD5, replate the cells on the coverslips coated with the extracellular-like matrix (**steps 8** and **9**) (*see Note 12*). To begin replating, first warm FM15 media to 37 °C and add 1 µg/mL DOX.
11. Add 200 µL 0.25% trypsin to each well of a 6-well plate (scale volume by $\frac{1}{2}$ for a 12-well plate) and return the plate to the incubator to trypsinize the cells at 37 °C for 2–3 min.
12. Check cells for dissociation. If still attached, return the plate to the incubator for an additional 30 s. You may also gently tap the plate against the palm of your hand to help dislodge the cells.
13. Once the cells are dissociated from the plate, quickly quench the trypsin with FM15 and 1 µg/mL DOX. For 200 µL trypsin, use 800 µL FM15 and scale by $\frac{1}{2}$ for a 12 well.
14. Transfer the resuspended cells into 1.5 mL Eppendorf tubes and centrifuge at 300 \times *g* for 5 min at room temperature.

15. During the centrifugation in **step 14**, take the plate containing coverslips and coating solution (from **step 9**) out of the incubator and aspirate the fibronectin/laminin coating solution from the wells. Try not to let the coverslips dry, as they now have an extracellular-like matrix for the cells to attach to.
16. After the centrifugation in **step 14**, aspirate the supernatant, leaving about 100 μ L behind to protect the cell pellet from being disturbed. Gently resuspend the pellet in the remaining 100 μ L. If you transduced an entire 6-well plate on PTD0, you will now have 6 microcentrifuge tubes (1 per well) with 100 μ L of cells each.
17. Cells may be replated on coated coverslips at different ratios. It is a good idea to start at 1:1 (one well of a 6-well plate to one 6-well coverslip) and 1:2 (one well of a 6-well plate to two 6-well coverslips) to determine the optimal density in your hands. Use approximately 160, 80, or 40 μ L for a 6-, 12-, or 24-well coverslip, respectively. For example, take two of the six microcentrifuge tubes from **step 16** and label them A and B. These both contain 100 μ L of cells from 1 well of a 6-well plate. To replate the cells in tube A at 1:1, add 60 μ L of FM15 containing 1 μ g/mL DOX, carefully mix, and drop all 160 μ L of cells onto a single 6-well coverslip. To replate the cells in tube B at 1:2, add 220 μ L of FM15 containing 1 μ g/mL DOX, carefully mix, and drop 160 μ L each onto two 6-well coverslips. Take care not to let the cells drip off the glass coverslip. Replate the cells in the 4 remaining microfuge tubes in a similar manner. Be sure to plan well in advance, so that you have enough coverslips prepared and can work in a timely fashion.
18. Return the plate to the incubator and incubate the cells for 45 min to 1 h until the cells attach.
19. Gently flood wells with FM15 + 1 μ g/mL DOX. We use 12 mL of media per plate. For a 6-well plate, use 2 mL per well and scale 12- and 24-well plates accordingly.
20. On PTD6, observe the cells under the microscope (Fig. 4, *see Notes 13–15*). After successful replating, most cells will be attached to the coverslips and there will be virtually no cell death. Then, aspirate the media from wells and replace with prewarmed Reprogramming Media I with the following small molecules: 1 μ g/mL DOX, 200 μ M cAMP, 1 mM VPA, 10 ng/mL BDNF, 10 ng/mL NT3, 1 μ M RA, 1 \times RVC, and 3 μ g/mL Puromycin (*see Notes 16–19*).
21. On PTD8, add 1000 \times DOX stock to a final concentration of 1 μ g/mL. Processes between cells are commonly observed by this day in reprogramming.
22. On PTD10, half-feed the cells. First remove slightly under $\frac{1}{2}$ volume (800 μ L for a well of a 6-well plate) and refeed with $\frac{1}{2}$ volume (1 mL per well). This will account for evaporation.

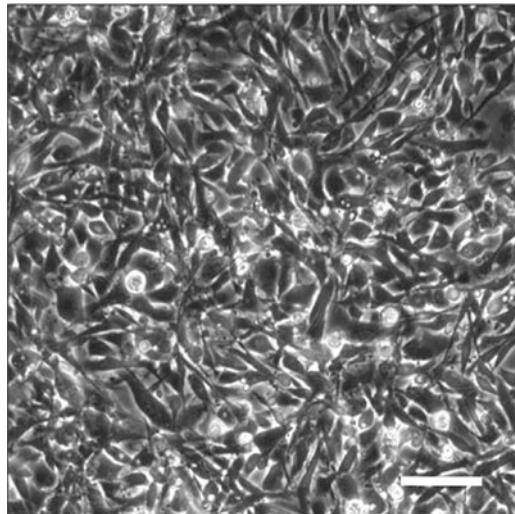


Fig. 4 Phase image of the cells on the day after replating (PTD6). Most cells are fully attached to the coverslip. Cells plated at high densities as seen in the picture promote better cell survival in long-term culture than cells plated at lower densities. Scale bar is 100 μM

Refeed with Reprogramming media I containing: 2 \times DOX (DOX at 2 $\mu\text{g}/\text{mL}$ so the final concentration after half-feeding will be 1 $\mu\text{g}/\text{mL}$) and 200 μM cAMP, 1 mM VPA, 10 ng/mL BDNF, 10 ng/mL NT3, 1 μM RA, 1 \times RVC, and 3 $\mu\text{g}/\text{mL}$ Puromycin.

23. On PTD12, add 1000 \times DOX to a final concentration of 1 $\mu\text{g}/\text{mL}$ per well. Cells will appear more neuronal, with more thin processes developing between cells.
24. On PTD14, cells will look like a classical neuron, having multiple neurites with very little branching. Half-feed the cells today as in **step 22**, using Reprogramming Media II instead of Reprogramming Media I. Be sure to include all of the small molecules at the same concentrations as PTD10 in **step 22**.
25. Repeat doxing and half-feeding cycles (**steps 23 and 24**) every 4 days so that cells are either doxed or half-fed every 2 days. We typically stop adding RVC after PTD14. DOX can be removed 4 days before analysis, or after PTD30 (*see Notes 20 and 21*).
26. When beginning neuronal reprogramming, it is key to first make sure miRNA-mediated reprogramming works in your hands. You can first assess morphological changes: cells will exit cell cycle by PTD2–PTD3 and neuronal morphologies will be clear by PTD14 and as early as PTD7 (Fig. 1). Once the cells have undergone clear morphological changes, it is essential to assess the expression of pan-neuronal genes from cells collected

around PTD15–PTD20 (*see Notes 22 and 23*). Only after the successful generation of miNs should one move on to subtype-specific reprogramming.

3.4 Generating Medium Spiny Neurons (MSN) Using Pro-neural Transcription Factors and miR-9/9^{*}-124

MSNs are a subtype of neurons that comprise 90–95% of neurons within the striatum, a part of the basal ganglia [18]. MSNs are the main cell type affected in HD, an inherited neurodegenerative disorder [19, 20]. Therefore, MSNs directly converted from fibroblast of HD samples have been shown to be instrumental for modeling HD. MSN-specific conversion is achieved by combining neurogenic activities of miR-9/9^{*}-124 with striatum-enriched transcription factors, CTIP2 (also known as BCL11B), DLX1, DLX2, and MYT1L [14, 16, 17]. CTIP2 and DLX1/2 have previously been shown to be important for differentiation of MSNs [21, 22]. MYT1L enhances direct neuronal reprogramming and neuronal maturation in conjunction with other factors [5, 23] and is also enriched in MSNs over other neuronal subtypes [24]. Under miR-9/9^{*}-124-directed neuronal conversion, the addition of CTIP2, DLX1/2, and MYT1L instructs a commitment to MSN fate and produces a highly enriched population of striatal MSNs characterized by the expression of MSN-specific markers, DARPP-32 (also known as PPP1R1B), DRD1, and DRD2 [21, 25, 26] as well as GABAergic markers GAD1 [16]. Electrophysiological properties of the reprogrammed MSNs that have been examined in monoculture, co-culture with rat primary glial cells and by transplantation into the mouse striatum suggest that the reprogrammed MSNs exhibit electrical membrane properties analogous to native MSNs [16].

To reprogram one plate of cells into Medium Spiny Neurons, follow the protocol for miNs with the following exceptions (*see Note 24*):

1. In step 4 of Subheading 3.3, use a mix of the following viruses: 504 µL miR-9/9^{*}-124, 504 µL rtTA, 504 µL MYT1L, 504 µL DLX1, 504 µL DLX2, and 124 µL CTIP2. Bring the volume to 24 mL with FM15 and 24 µL of 8 mg/mL (or 1000×) PB.
2. On PTD 3, 6, and 10, add Blasticidin to a final concentration of 3 µg/µL, G418 to a final concentration of 300 µg/µL, and Puromycin to a final concentration of 3 µg/µL. Continue adding puromycin only in half-feedings until PTD30 (*see Notes 25 and 26*).

3.5 Generating Motor Neurons Using Pro-neural Transcription Factors and miR-9/9^{*}-124

miNs can also be guided to a spinal cord motor neuron fate with the use of subtype-specific transcription factors. ISL LIM homeobox 1 (ISL1) and LIM homeobox 3 (LHX3) are conserved developmental transcription factors that synergize to specify spinal cord motor neuron fate [27, 28]. When ISL1 and LHX3 are co-expressed with miR-9/9^{*}-124 to guide the neuronal conversion

towards the motor neuron (MN) lineage, key marker genes of MNs are activated, including *MNX1*, *CHAT*, *VACHT*, *LMO1*, and *LMO4* [9]. In addition to the extensive activation of motor neuron transcription networks, the converted MNs demonstrate functional maturity through complex electrophysiological features when compared to miNs alone and have the capacity to form neuromuscular junctions on myotubes *in vitro* [9].

To reprogram one plate of cells into MNs, follow the protocol for miNs with the following exceptions (*see Note 24*):

1. In **step 4** of Subheading 3.3, use a mix of the following viruses: 504 μL miR-9/9*-124, 504 μL rtTA, 504 μL ISL1, 504 μL LHX3. Bring the volume to 24 mL with FM15 and add 24 μL of 8 mg/mL (or 1000 \times) PB.
2. On PTD 3, 6, 10, and 14, add G418 to a final concentration of 300 $\mu\text{g}/\mu\text{L}$, in addition to Puromycin to a final concentration of 3 $\mu\text{g}/\mu\text{L}$. Continue adding puromycin only in half-feedings until PTD30.

3.6 Generating Cortical Neurons Using Pro-neural Transcription Factors and miR-9/9*-124

Cortical miNs were first described in the seminal paper “Micro-RNA-mediated conversion of human fibroblasts to neurons.” Primarily glutamatergic miNs were generated through the supplemental expression of the transcription factors NEUROD2, ASCL1, and MYT1L. MYT1L is a pro-neural factor expressed in multiple neuronal subtypes that increases neuronal maturity with no detriment to neuronal survival [17]. NEUROD2 is an important basic helix-loop-helix (bHLH) transcription factor whose activity in the brain is vital for cellular differentiation [29, 30]. ASCL1 is another bHLH transcription factor whose activity can push the conversion towards the GABAergic and neuronal progenitor fate [23, 29]. However, we consistently find that expressing too much bHLHs such as NEUROD2 and ASCL1 can lead to genotoxicity and their expression levels need to be carefully controlled. With this in mind, we currently use only NEUROD2 and MYT1L for enriching excitatory cortical neurons.

To reprogram one plate of cells into cortical neurons, follow the protocol for miNs with the following exception (*see Note 24*):

1. In **step 4** of Subheading 3.3, use a cocktail of the following viruses: 504 μL miR-9/9*-124, 504 μL rtTA, 504 μL MYT1L, and 63 μL NEUROD2. Bring the volume to 24 mL with FM15 and add 24 μL of PB.
2. We currently do not find it necessary to select with antibiotics other than puromycin as detailed in Subheading 3.3 when making cortical neurons as we use MYT1L and NEUROD2 under the same selectable marker. Selection does not alter cell number, nor does it increase cortical gene expression.

4 Notes

1. Healthy 293LE cells are crucial for the production of high-quality lentiviruses. It is important to prevent 293LEs from becoming over-confluent, to passage them regularly and not too sparsely, and to use low passage (<P12–P13) cells that are 80–90% confluent at the time of transfection.
2. PEI is very sensitive to pH and can easily aggregate or precipitate out of solution. To ensure high-quality PEI, be careful not to overshoot the pH when adjusting it with NaOH (Subheading 2.7). Readjusting an overshot pH with addition of acid will form salt and reduce the transfection efficiency. Because of this pH sensitivity, it is very important to add PEI last when making the transfection master mix (step 2, Subheading 3.1).
3. When you are working with lentivirus, wear a lab coat and gloves. Everything that touches the lentivirus must be soaked in 10% bleach before disposal in the biohazard waste.
4. Virus is concentrated and purified as explained in the methods. If you do not have access to a high speed or ultracentrifuge, it is possible to use the non-concentrated supernatant (roughly 10× the volume of concentrated virus). We have found this to work well for some cell lines; however, others are sensitive to the increased pH and debris from 293LEs. On the other hand, using the supernatant directly can save time and reagents and is a skill that can be learned and used on a case-by-case basis.
5. When using supernatant instead of concentrated virus you may see debris on PTD1 that was not noticeable post-spinfection on PTD0 (Fig. 5). The amount of debris may vary depending on the cell line and viruses used. One wash with DPBS is sufficient to remove the debris and the cells reprogram normally.
6. After high-speed centrifugation, concentrated virus is found in a transparent pellet, often with a brown tint. The pellet is likely a combination of virus and vesicles/debris excreted from the 293LEs. If you are experiencing toxicity of the virus as measured by fibroblast death/debris before antibiotic selection, you may consider using a sucrose gradient to obtain a more highly purified virus [31].
7. Nitric acid is corrosive and its vapor can be harmful.
8. Fibroblasts should be fully confluent on PTD-1 and replated to be fully confluent the next day at transduction (*see* Fig. 1, PTD0). Replating from a plate that is not confluent on PTD-1 may reduce transduction and reprogramming efficiency, which will manifest as cell death/debris before antibiotic selection on PTD3. Similarly, transducing cells that are not fully confluent on PTD0 may result in cell death before PTD3 (Fig. 6b) and ultimately decreased reprogramming efficiency.

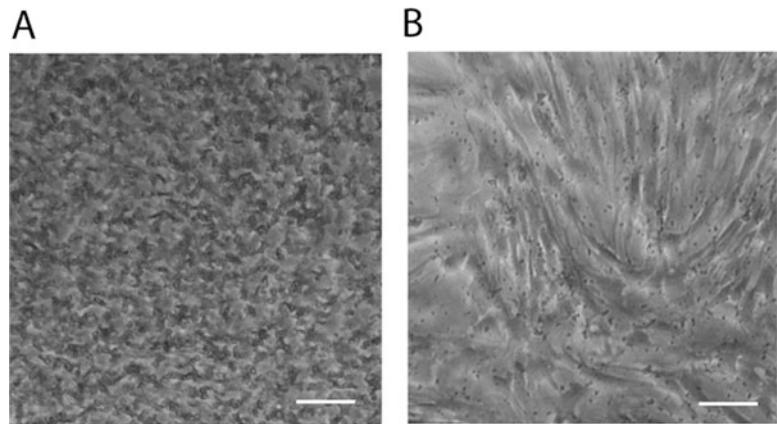


Fig. 5 Cells transduced on PTD0 with viral supernatant rather than concentrated virus may exhibit debris floating in the media on PTD1 (**a**). A PBS wash on PTD1 clears most of the debris (**b**) and the cells reprogram normally. Scale bars are 100 μM

9. Doubling time is a key indicator of fibroblast reprogramming capacity. Typically, fibroblasts that double in roughly 48–72 h or less reprogram well. In contrast, slow growing fibroblasts often do not reprogram well, and this is evident as early as day 2–3 by the presence of cellular debris/death (Fig. 6b). Additionally, fibroblasts prefer to be split at low ratios once confluent (1:2–1:4). If plated too sparsely, they tend to increase their doubling time prematurely.
10. We often perform secondary transductions with additional factors that are not involved in neuronal or subtype conversion (e.g., GFP). In such cases, transductions may be performed on either PTD2–4 or PTD10–14. PTD2–4 transduction may be performed in the same manner as PTD0 transduction (with spinfection and polybrene) and is appropriate for factors that will not interfere with reprogramming or that need to be expressed at an earlier time point. If the additional factors are believed to interfere with reprogramming or are not needed until a later time point, we transduce around PTD10–14. In this case, concentrated virus is applied directly into the media, as spinfection and polybrene will kill the neurons. There is no need to change the media the next day (in fact, later stages of reprogrammed neurons do not like having media changed, apart from half-feedings).
11. We typically replate on 24-well coverslips for microscopy, 12- or 6-well coverslips for RNA extractions and 6-well coverslips for preparation of protein lysates. There is also an option to replate on tissue culture plates that have a modified polystyrene surface (called Corning Primaria, see materials list) and do not require glass coverslips. In our experience, these plates are

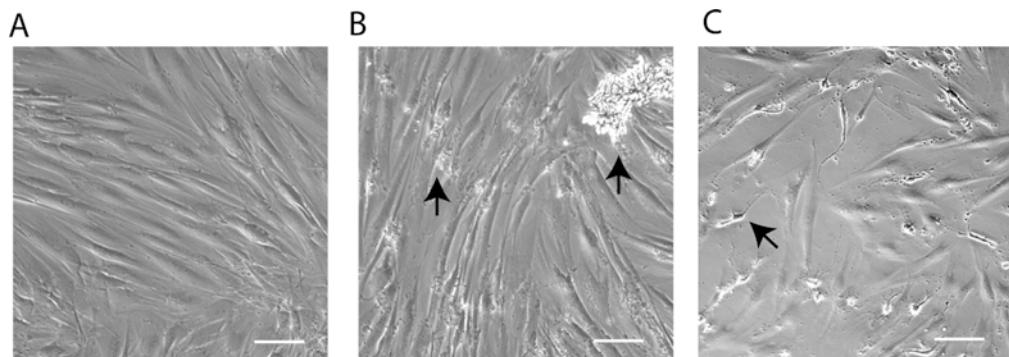


Fig. 6 (a) Healthy PTD3 cells before puromycin selection. (b) PTD3 cells exhibiting cell death and debris (arrowheads) before antibiotic selection. Cell death before antibiotic selection is often due to too high viral titer. (c) PTD4 cells exhibiting cell death after antibiotic selection. Death after antibiotic selection is often due to too low viral titer. Healthy PTD4 cells resemble cells in (a). Scale bars are 100 μM

good for time points up until around PTD20. Past this time point, cells may begin popping off the dish. When large quantities of cells are needed, or when we are assaying cells PTD20 or earlier, Primaria plates offer an alternative option.

12. Once you are familiar with the reprogramming protocol, you can modify it in several ways to simplify the protocol or to meet your specific needs. For instance, replating can be done on PTD5, PTD6, or PTD7. To replate on either PTD6 or PTD7, begin on PTD5 by aspirating the media and replacing it with FM15 containing 1 $\mu\text{g}/\text{mL}$ DOX, 1 \times RVC, 3 $\mu\text{g}/\text{mL}$ Puromycin, and any subtype-specific antibiotics. Then, wait until either PTD6 or PTD7 (your preference) and begin the replating protocol in **step 10**, Subheading **3.3**. Continue the protocol from here on.
13. Puromycin selection begins on PTD3. If lentivirus transduction was efficient, cells will maintain their starting density in the presence of antibiotics (*see* Fig. 1 and compare the density of PTD0 and PTD5 cells). If cell death is observed on PTD4 (after antibiotic selection, Fig. 6c), the miR-9/9^{*}-124 or rtTA virus titer may have been too low, the lentivirus may have had poor overall quality, or you may have forgotten to dox the cells beginning on PTD1. When there is substantial cell death this early in reprogramming, the remaining cells typically do not reprogram well and it is not worth proceeding with the reprogramming protocol.
14. After replating, cells form a monolayer (Fig. 4). In some instances, you may see a few bright white cells that look like they are not attached to the coverslips; this is normal. If many of the cells look this way, it can be a sign that the cells were replated too densely, so there is not enough space for the cells

to form a monolayer. In this case, when you replate for the next experiment you may try replating with a more dilute ratio (e.g., 1:2 instead of 1:1 in **step 17** of Subheading **3.3**). In contrast, if the cells were plated too sparsely you may try decreasing the ratio.

15. If cells begin to die after replating (PTD5–PTD6), this is an indication of harsh treatment during replating, or that you plate too slowly. When you are first learning how to reprogram, it is a good idea to replate one plate at a time. Work quickly and be gentle while pipetting cells during the replating process.
16. It was recently shown that VPA, a small molecule used in this reprogramming protocol, is toxic for neurons differentiated directly from ES cells [32]. In contrast, VPA is essential for successful miRNA-mediated reprogramming of fibroblasts into miNs. Cultures of miNs reprogrammed in the absence of VPA are sparse and short-lived (Fig. 7).
17. VPA is toxic, avoid ingestion and contact with skin. Avoid breathing dust formation. Use personal protective equipment, including gloves, mask, eye protection, and a lab coat.
18. RA is toxic, avoid ingestion and contact with skin. Use personal protective equipment such as gloves, mask, eye protection, and lab coat.
19. The choice of media after replating has some flexibility. We have successfully reprogrammed cells in NeuroBasalA (Gibco), BrainPhys (Stemcell), or Neuronal Media (ScienCell). Each medium has its benefits and drawbacks. NeurobasalA helps the cells survive for more than a month in culture, but the neurons tend to be less mature. BrainPhys helps neurons to mature, but cells tend to live shorter. When cultured in Neuronal Media cells reach maturity while also surviving a long time in culture and many beginners find reprogramming to be easiest in this media. Unfortunately, we have experienced variability in the batch to batch consistency of Neuronal Media and this must be kept in mind when purchasing a new lot.
20. RVC is removed around PTD14; however, we have found that adding it during half-feedings until PTD30 improves cell morphology. Some people therefore prefer to add it to the cells that will be used for microscopy. Note that the cells will mature faster and as a result live less time in culture.
21. Puromycin may be removed before PTD30 and as early as PTD14 if the cells are already very neuronal. This may be useful if you think puromycin will interfere with your downstream assays.

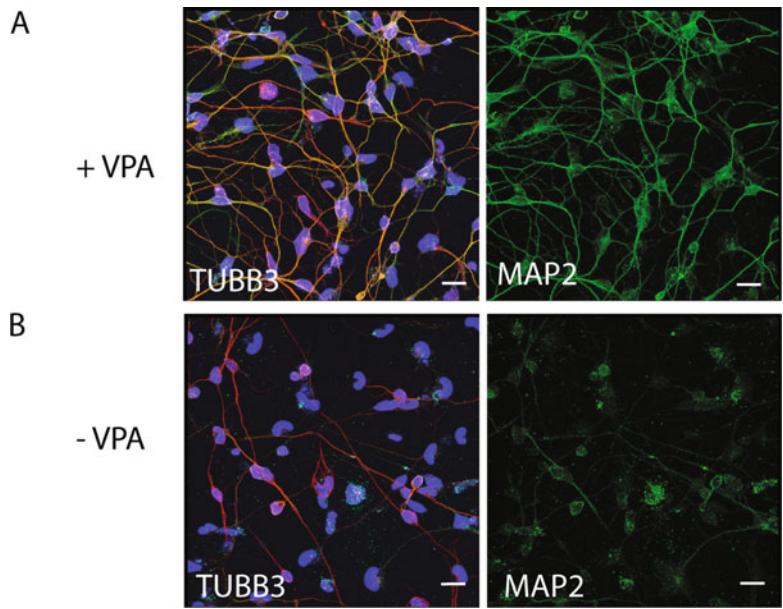


Fig. 7 PTD30 miNs cultured with VPA (**a**) and without VPA (**b**). While VPA has been shown to be toxic to neurons reprogrammed from ES cells [32], it is required for miRNA-mediated reprogramming of fibroblasts. Cells are stained with Tuj1 (red in merge, left), DAPI (blue in merge, left) and Map2 (green, right). Scale bars are 20 μ M

22. Not all fibroblasts reprogram with the same efficiency. Before comparing results from neurons reprogrammed from multiple cell lines, it is imperative to check reprogramming efficacy for markers of neuronal maturity as measured by immunohistochemistry or RT-qPCR. In both cases, fibroblasts are used as a negative control. Efficiency of miRNA-mediated reprogramming can also be examined by checking both the downregulation of fibroblast-enriched genes (i.e., *S100A4*, *S100A3*, *VIM*, *MEAP5*, *COL1A1*, *ITGB1*, *PAMR1*, *HSPB7*) and upregulation of neuronal genes (i.e., *MAP2*, *NCAMI*, *NEFL*, *SNAP25*, *PSD95*, *DLG4*, *DPF1*, *BEX2*, *HOOK1*, *CNTN1*, *RBFox3*) [9].
23. To assess functional maturity, expression of long genes (>100 kb long) may be assayed by RNA-seq in comparison to the starting human fibroblasts as the enrichment of long genes is a transcriptomic feature unique in neurons as described previously [15]. If RNA-seq is not feasible, one could test the expression of select long genes (but not limited to) including *DLG2*, *CDH18*, *PCDH9*, *DGKB*, *RIMS2*, *ANK3*, *KALRN*, *SYTL*, *DCLK2*, *KIF1A*, *DNER*, and *NCAMI*. If reprogrammed cells display gene expression profiles that support

neuronal conversion, the converted neurons should also be analyzed by the whole-cell recording to assess the cells' electrophysiological properties.

24. Subtype reprogramming efficiency is also assessed through immunocytochemistry (Fig. 8) or qPCR; MSNs express DARPP32, DRD2, and GAD1 [14, 16, 17]; MNs express CHAT and MNX1 [9]; and CNs express VGLUT1, TBR1, and SATB2 [5].

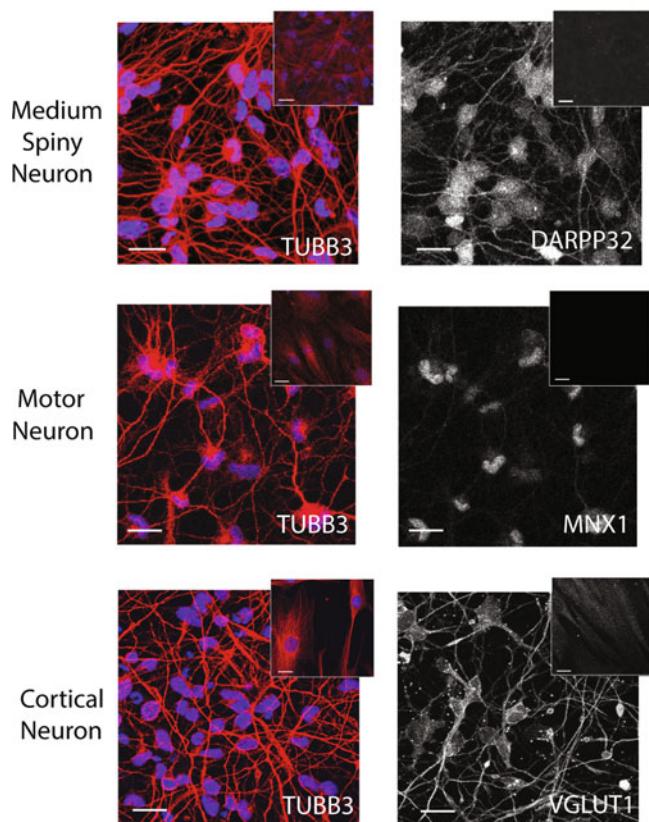


Fig. 8 Mature Medium Spiny, Motor and Cortical Neurons derived from human fibroblasts via miRNA-mediated direct conversion (PTD28 or older). Subtype-specific reprogramming is assessed by staining for the MSN marker DARPP32 (rabbit anti-DARPP32 Cat #sc-11365 from Santa Cruz), motor neuron marker MNX1 (mouse anti-MNX1 from Developmental Studies Hybridoma Bank (DSHB Cat# 81.5C10)), and cortical neuron marker VGLUT1 (mouse anti-VGLUT1 Cat# 135311 from SySy) (grey, right panel). TUBB3 staining visualizes cell morphology (red, left panel). Nuclei are visualized in blue using DAPI/Hoechst. Subtype-specific markers are expressed in reprogrammed neurons but not fibroblasts (insets show fibroblast controls). Scale bars are 20 μ M

25. Some people find MSN reprogramming to be more challenging than miN, cortical neuron, or MN reprogramming. The most important thing for MSN reprogramming is to get a consistent titer for each virus. Measuring the titer may be helpful until you establish a consistent method to generate each virus. More details on virus titration can be found in Richner et al., Nat Protoc, 2015 [17].
26. We have noticed that reducing the number of viruses used during MSN reprogramming may lead to healthier cells. If you are having difficulty specifically with MSN reprogramming, it is possible to construct a plasmid that dually expresses DLX1 and DLX2, using P2A as a self-cleaving peptide, thereby reducing the number of viruses required.

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Chapter 7

Reprogramming Human Fibroblasts to Induced Pluripotent Stem Cells Using the GFP-Marked Lentiviral Vectors in the Chemically Defined Medium

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Abstract

Much investigation is needed to understand the underlying molecular mechanisms of iPSC reprogramming and to improve this technology. Lentivirus-mediated iPSC reprogramming remains the most effective method to study human pluripotency reprogramming. iPSC production is more efficient and consistent in the chemically defined medium. Fibroblasts are the most common starting cells for iPSC generation. Here, we provide a detailed protocol for iPSC generation from human fibroblasts using the GFP-expressing lentiviral vectors in the chemically defined medium.

Key words Human induced pluripotent stem cells, Human iPSC, Fibroblasts, Lentiviral vectors, Chemically defined medium, Pluripotent reprogramming, Co-expression of GFP

1 Introduction

Human somatic cells can be converted into pluripotent stem cells (PSCs) by ectopic expression of several reprogramming factors [1, 2]. Such factor-induced PSCs (iPSCs) from patient somatic cells have great potentials in regenerative medicine because human iPSCs (HiPSCs) have the same properties as human embryonic stem cells do. iPSC reprogramming is still inefficient, slow, stochastic, incomplete, and aberrant [3] although significant improvements have been made over the last 13 years. The molecular mechanisms for iPSC reprogramming remain a black box to scientists. Much research is needed to understand this process and to improve the iPSC technology.

Various protocols have been developed to generate human transgene-free iPSCs using non-integrating methods [4–6], but in the research setting virus-based iPSC reprogramming remains the most effective approach for investigation of iPSC reprogramming because of the low efficiency and technical limitations of the

non-integrating systems [7–9]. Human fibroblasts are the most common starting cells for iPSC generation because of their easy access and simple maintenance. Here, we provide a protocol to reprogram human fibroblasts into iPSCs in the chemically defined medium using the lentiviral vectors to deliver the reprogramming factors. We use the chemically defined medium because it is more efficient and consistent [10]. The defined medium will make it easier to screen for new reprogramming genes and reprogramming-enhancing chemicals [8, 9]. We use the lentiviral constructs with the EF1 α promoter to drive expression of the reprogramming factors and co-expression of GFP mediated by the P2A peptide [6, 9]. Use of the EF1 α promoter avoids premature silencing of the reprogramming factors as compared to the CMV promoter. The vector design with a GFP co-expression with the reprogramming factors has the following advantages: (1) the functional titers of the reprogramming viruses can be determined easily using flow cytometry. This is critical because proper stoichiometry of the reprogramming factors is an important parameter for efficient reprogramming. (2) The transfection and transduction efficiency can be monitored readily using flow cytometry and/or fluorescent microscopy. (3) Silencing status of the transgenes in the iPSC colonies can be judged readily by the levels of GFP expression. Silencing of the integrated reprogramming factors in the reprogrammed iPSCs is a hallmark of complete reprogramming. Because there is a GFP co-expression with each of the three reprogramming factors in our constructs (Fig. 1a), lack of expression for multiple copies of GFP transgenes integrated in the generated iPSCs will effectively indicate sufficient silencing for all of the transgenes.

This chapter will provide step-by-step procedures for: (1) packaging of the reprogramming lentiviral particles (Subheading 3.1); (2) Concentration of the reprogramming lentiviral particles produced (Subheading 3.2); (3) functional titration of the reprogramming viruses (Subheading 3.3); (4) generation of human iPSCs from fibroblasts in the chemically defined medium using the lentiviral reprogramming cocktails (Subheading 3.4); and (5) expansion/maintenance and long-term storage of the established iPSCs (Subheading 3.5). Our reprogramming protocol uses only three of the four Yamanaka factors since MYC does not have any beneficial effect on iPSC reprogramming in the chemically defined medium [9, 11].

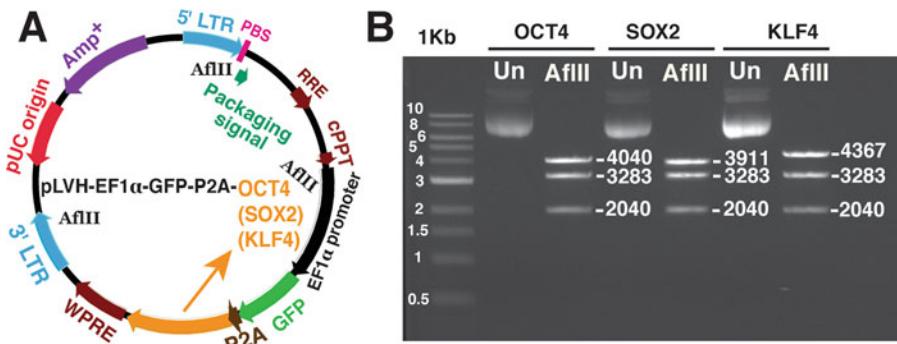


Fig. 1 Lentiviral reprogramming factors with co-expression of GFP. **(a)** Schematic map for the lentiviral reprogramming constructs with GFP co-expression mediated by a P2A peptide. *LTR*, long terminal repeat; *PBS*, primer binding sequence; *RRE*, Rev response element; *WPRE*, woodchuck hepatitis virus post-transcriptional regulatory element; *cPPT*, central polypurine tract; *EF1 α* , human *EF1 α* promoter; *pLVH*, lentiviral vector plasmids in Hu laboratory. The locations of the three *AflIII* sites are indicated. **(b)** Confirmation of plasmid integrity for the lentiviral reprogramming constructs. The sizes for each fragment in base pair (bp) are indicated in the gel image. The fragment sizes in kilobase pair (kb) of the 1 kb marker are also indicated. The quality of the plasmids can also be judged by the predominant bands for the supercoiled plasmids without restriction digestion (*Un* lanes). *Un*, uncut

2 Materials

All reagents should be cell culture grade. Aseptic practice should be observed for all steps. Users of this protocol should have received specific training in handling pathogenic agents. This protocol should be conducted in a biosafety level 2 (BSL2) laboratory because lentiviral vectors are used.

2.1 Reagents

1. Lenti-X 293T, for lentivirus packaging.
2. HeLa cells, for lentiviral titration.
3. Hexadimethrine bromide (Polybrene).
4. Human foreskin BJ fibroblasts or other human fibroblasts as starting cells for iPSC generation.
5. TrypLE.
6. hESC-qualified Matrigel.
7. ROCK inhibitors, Y-27632 or thiazovivin.
8. Polyethylene glycol 6000 (PEG-6000).
9. Tris-HCl buffer: 50 mM Tris, pH 7.4.
10. Bleach.
11. Maxiprep Kits.

2.2 Plasmids

1. Envelope plasmid pMD2.G (*see Note 1*).
2. Packaging plasmid psPAX2.

3. pLVH-EF1 α -GFP-P2A-OCT4 (Addgene, cat# 130692) (Fig. 1a) (*see Note 2*).
4. pLVH-EF1 α -GFP-P2A-SOX2 (Addgene, cat# 130693).
5. pLVH-EF1 α -GFP-P2A-KLF4 (Addgene, cat# 130694).

2.3 Key Equipment

1. Centrifuge with capacity of refrigeration.
2. Flow cytometer.
3. -80 °C freezer.
4. Freezing container for initial freezing of iPSCs.
5. Liquid nitrogen storage tank for long-term storage of iPSCs.
6. CO₂ incubator (*see Note 3*).
7. Laboratory biosafety cabinet.
8. Nanodrop.
9. Sharp container.
10. Personal protective equipment (PPE): gloves, masks, laboratory coats, and safety goggles.
11. Portable liquid-N₂ tank or styrofoam box for snap-freezing of lentiviral preparations.
12. Test-tube cooler for thawing Matrigel inside a 4 °C refrigerator.
13. 0.2-μm filters for sterilization of culture media.
14. 0.45-μm filters with receiver flasks, for virus collection.
15. FACS tubes with cell strainer caps, strainer pore size 35 μM.

2.4 Media

1. Fibroblast growth medium: Dulbecco's Modified Eagle Medium (DMEM) with high glucose, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.1 mM 2-mercaptoethanol, 1× penicillin-streptomycin (100 U/mL penicillin, and 100 μg/mL streptomycin), 0.1 mM Minimum Essential Medium (MEM) Non-Essential Amino Acids (NEAA), and 4 ng/mL human basic fibroblast growth factor (bFGF, also known as FGF2) (*see Note 4*).
2. E8 medium (pH 7.4) for iPSC maintenance: DMEM/nutrient mixture F-12 (F-12) (DMEM/F-12), 1.74 g/L NaHCO₃ (*see Note 5*), 64 mg/L L-ascorbic acid 2-phosphate sesqui-magnesium (*see Note 6*), 13.6 μg/L sodium selenium, 4 ng/mL FGF2, 20 μg/mL insulin, 10 μg/mL transferrin, and 2 μg/L TGFβ1 (*see Note 7*).
3. 293T growth medium: DMEM, 10% FBS, 1× MEM Non-Essential Amino Acid (MEM NEAA), 1× penicillin-streptomycin (100 U/mL penicillin and 100 μg/mL streptomycin).

4. HeLa growth medium: DMEM, 10% FBS, 1× penicillin-streptomycin (100 U/mL penicillin and 100 µg/mL streptomycin).
5. Reprogramming E7 medium: E8 medium without TGFβ1, and with addition of sodium butyrate at the final concentration of 100 µM.
6. iPSC freezing medium: E8 medium supplemented with 10% dimethyl sulfoxide (DMSO).

2.5 Coated Culture Dishes/Plates

1. Matrigel-coated dishes for reprogramming or iPSC passaging: Take one aliquot of Matrigel from the –80 °C freezer one day before use, and thaw the Matrigel overnight inside a portable test-tube cooler inside a 4 °C refrigerator or on ice in a small ice box with a cover inside a 4 °C refrigerator. Prepare the diluted solution of Matrigel on ice with ice-cold DMEM/F-12 media at the concentration of 75–150 µg/mL (*see Note 8*). Add 2.5 mL of the diluted working Matrigel solution into a 60-mm dish and incubate at 37 °C at least for 30 min before use. Just before use, take the dish with coating Matrigel solution out of the 37 °C incubator, remove the Matrigel solution as completely as possible by aspiration, and immediately add the appropriate amount of media containing the iPS cells or the reprogramming fibroblasts (*see the procedure below*).
2. Collagen-coated dishes for lentiviral packaging: Dissolve collagen I powder in 0.02 N acetic acid solution to a final concentration of 50 µg/mL collagen. Add 15 mL of the collagen-I solution into one 150-mm tissue culture dish and incubate for 30 min at 37 °C (5 µg/cm²). Remove the solution (*see Note 9*) and wash the dish with 12 mL of DPBS. Remove as much as possible of DPBS liquid using a Pasteur pipet attached to a vacuum line, and then dry the coated dish by incubating at 37 °C for 1–2 h before use.

2.6 Buffers and Solutions

1. 2× BES-buffered saline (BBS), pH 7.07: 50 mM *N,N*-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 280 mM NaCl, and 1.5 mM Na₂HPO₄·7H₂O. Mix 16.36 g of NaCl, 10.65 g of BES, and 0.402 g of Na₂HPO₄·7H₂O. Add pure water up to 900 mL. Dissolve the chemicals, titrate to pH 7.07 with 1 M NaOH at room temperature, and bring the volume to 1 L with pure water. Sterilize by filtration using a 0.2-µm filter. Aliquot into 15-mL sterile tubes and store the BBS aliquots at –20 °C.
2. 2.5 M CaCl₂ solution: Dissolve 36.75 g CaCl₂·2H₂O in 100 mL ultrapure water. Sterilize with a 0.2-µm membrane filter. Aliquot at 10 mL/tube and store the aliquots at –20 °C.

3. Tris Buffered Saline (TBS) for storage of concentrated viruses: 50 mM Tris-HCl, 150 mM NaCl, pH 7.4.
4. EDTA iPSC-dissociation solution: 0.5 μM ethylenediaminetetraacetic acid (EDTA), 0.18% NaCl (w/v) in PBS. Add 1 mL of 0.5 M EDTA (pH 8.0), and 1.8 g of NaCl into 1 L of calcium/magnesium-free PBS. Sterilize the solution by autoclave or by filtration. Store at room temperature or at 4 °C.
5. Fluorescence-Activated Cell Sorting (FACS) buffer: FACS buffer is the standard PBS buffer (without calcium and magnesium) supplemented with 2% FBS (v/v), 2.5 mM EDTA, and 0.05% sodium azide. Sterilize the buffer by filtration using 0.2-μm membrane filters.

3 Methods

3.1 Packaging Reprogramming Lentiviruses (See Note 10)

1. Prepare high-quality plasmids using a Maxiprep kit as instructed by the manufacturer for all of the five plasmids (*see Note 11*). Measure the concentration and evaluate the quality of the plasmid preparations using Nanodrop. Make sure the transfer plasmids are intact by running a standard agarose gel of the digested plasmids with the restriction enzyme AflII (Fig. 1b) (*see Note 12*).
2. Into one collagen I-coated 150-mm dish, seed 1.5×10^7 Lenti-X™ 293T cells in 25 mL of the 293T growth medium, and culture at 37 °C, 5% CO₂, for 24 h.
3. The next day, replace the spent media with 20 mL of pre-warmed fresh 293T growth media 1–3 h prior to transfection.
4. For a 150-mm dish, prepare 60 μg of total plasmid DNA mix at a ratio of 10.5 μg envelop plasmid pMD2.G, 19.5 μg packaging plasmid psPAX2, and 30 μg transfer vector (pLVH-EF1-α-GFP-P2A-OCT4, pLVH-EF1α-GFP-P2A-SOX2, or pLVH-EF1α-GFP-P2A-KLF4), and mix with 150 μL of the 2.5 M CaCl₂ solution (*see Note 13*). Bring the DNA/calcium solution to 1.5 mL using the ultrapure water and mix well in a sterile 15-mL tube.
5. Mix the DNA/calcium preparation with an equal volume (1.5 mL each, 1:1) of 2× HBS (pH 7.07); pipette 15–25 times gently using a 5-mL pipette.
6. Immediately, add the DNA complexes dropwise into the cell culture media and swirl gently to mix with the culture media.
7. Incubate the cells for 16–18 h at 37 °C in 5% CO₂.

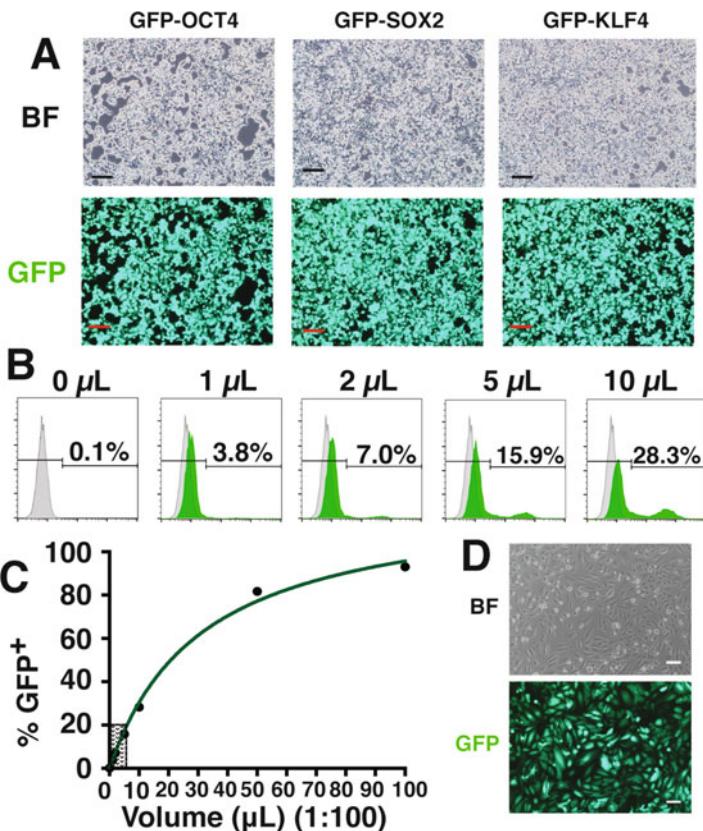


Fig. 2 Viral preparation and titration of the lentiviral reprogramming factors. **(a)** Representative Bright Field (BF) and Fluorescence (GFP) microscopy images of HEK293T cells, 24 h post transfection with the three lentiviral reprogramming factors as indicated. Scale bar = 50 μM . **(b)** Representative histograms of the titration analyses by flow cytometry of HeLa cells. Four different virus volumes from the 1:100 dilution preparation were analyzed along with an uninfected control. **(c)** Saturation curve model of percentage of infected cells versus volume of viruses from the 1:100 dilution preparation. The highly linear region below 20% of GFP⁺ cells (boxed) should be used for titer calculation. **(d)** Bright Field (BF) and Fluorescence (GFP) microscopy images of HeLa cells transduced with 1 μL of concentrated viruses of the OCT4 reprogramming factor. Scale bar = 100 μM

8. After 16–18 h, remove the transfection media, and then gently add 25 mL of fresh 293T growth media to the dish. Continue to culture the cells at 37 °C, 5% CO₂. After 24 h post transfection, check the transfection using fluorescence microscopy. Make sure the transfection efficiency is greater than 90% in order to achieve a high titer (Fig. 2a).
9. Collect the virus-containing media between 48 and 72 h post transfection.

3.2 Concentration of Lentiviral Particles by PEG Precipitation

1. Centrifuge the lentivirus-containing media collected above at $3000 \times g$ for 10 min at 4 °C to remove the cells debris, and then further clear the viruses of cell debris by filtration using 0.45-μm filters.
2. Transfer the viral supernatants to a new sterile 50-mL centrifuge tube, then add the appropriate amounts of 50% PEG-6000 stock solution and 4 M NaCl solution so that a final concentration of 8.5% PEG-6000, and 0.4 M NaCl will be obtained (*see Note 14*).
3. Precipitate the viruses at 4 °C for 3–5 h. Mix the viruses every 20–30 min.
4. Centrifuge at $3,900 \times g$ for 30 min to 1 h at 4 °C (*see Note 15*).
5. Carefully decant the supernatant and add pre-cooled TBS at 1% the volume of the original virus-containing media (100× dilution) into the tube (*see Note 16*).
6. Resuspend the viral pellets by pipetting up and down, and then vortex the tubes vigorously for 20–30 s.
7. Fill a portable liquid-N₂ tank (or a styrofoam container or an ice bucket) with liquid N₂.
8. Using aerosol-resistant filtered sterile pipette tips to transfer the lentiviral suspension into 500-μL microcentrifuge tubes in an aliquot of 10 μL each, and immediately throw each viral aliquot into liquid N₂ prepared at step 7 above (*see Note 17*).
9. Store the viruses at –80 °C immediately after completion of aliquoting.

3.3 Functional Titration of Lentiviral Particles by Flow Cytometry

1. Seed HeLa cells using the Hela growth medium into one 60-mm tissue culture dish. Culture cells until confluence.
2. Passage the cells into at least 28 wells of 24-well plates at a density of 2.5×10^4 cells per well.
3. The next day, quantify the cell number from two of these wells. First, detach the cells with 250 μL per well of TrypLE for 3 min. Stop the reaction with 250 μL of the Hela growth medium. Combine the cell suspension from the two wells (final volume of 1 mL), mix well, and take 10 μL to count the cells with a hemocytometer. Divide the calculated cell number by 2 in order to obtain the cell number per well. Keep a record of the cell number per well for titer calculation later.
4. Thaw one 10 μL-aliquot per lentiviral vector (pLVH-EF1-α-GFP-P2A-OCT4, pLVH-EF1α-GFP-P2A-SOX2, and pLVH-EF1α-GFP-P2A-KLF4) on ice. Take 5 μL of concentrated viruses and dilute with 495 μL of PBS (dilution factor = 100). Mix well and keep the viruses on ice until use (*see Notes 18 and 19*).

5. Add 15 mL of the HeLa medium in a 50-mL falcon tube and add Polybrene at 4 µg/mL.
6. Remove the medium from the plate with the HeLa cells and then transfer 500 µL of the Polybrene-supplemented medium to each well.
7. Use four different volumes of the diluted virus: 1, 2, 5, and 10 µL per well with two replicates (8 wells per reprogramming factor). Leave at least 1 well un-transduced as a control for flow cytometry analysis. Mix well (*see Note 18*).
8. Incubate the plate overnight at 37 °C in 5% CO₂.
9. The next day, remove the medium carefully and replace it with fresh HeLa medium.
10. On day 3, observe the cells using fluorescent microscope and make sure the Hela cells are expressing GFP (Fig. 2d). After 48 h, remove the medium and detach the cells by TrypLE treatment for 3 min at 37 °C.
11. Inactivate the reaction with 1 mL of FACS buffers or the Hela growth medium. Filter the cells into round-bottom tubes with a cap cell strainer.
12. Centrifuge at 400 × g for 5 min at 4 °C.
13. Discard the supernatant and resuspend the cell pellet with 1 mL of FACS buffer.
14. Run flow cytometry to analyze for the percentage of GFP-positive cells.
15. Analyze the data using a flow cytometry software such as FlowJo (Fig. 2b). Consider only the values of two or more volumes that fall between 2% and 20% of GFP-positive cells (Fig. 2b, c) (*see Note 20*).
16. Calculate the titer in Transduction Units per milliliter (TU/mL) using the following formula:

$$\text{TU/mL} = \frac{N \times F_{\text{GFP}} \times D_f \times 1000}{V_{\mu\text{L}}}$$

$V_{\mu\text{L}}$ = volume of diluted virus in µL.

N = cell numbers at the time of transduction.

F_{GFP} = fraction of GFP-positive cells expressed as percentage.

D_f = is the dilution factor. In this case, it is 100 (*see Note 19*).

3.4 Conversion of Human Fibroblast into iPSCs

1. Culture human foreskin BJ fibroblasts in the fibroblast medium (*see Note 21*).
2. Seed BJ fibroblasts into a 6-well plate at 1×10^5 cells per well.

3. Twenty-four hours post plating, premix the OSK viruses (OCT4 at 8 Multiplicity Of Infection (MOI); SOX2 at 5 MOI; KLF4 at 5 MOI), and add the viral cocktails into the cells to be reprogrammed using fibroblast medium supplemented with 4 µg/mL of Polybrene (*see Note 22*). For an accurate transduction, quantify the cell numbers at the time of transduction (N) from 1 well of the same plate. Calculate the required amount of viruses using the following formula: µL of viruses = $\frac{N \times \text{MOI} \times 1000}{\text{TU}}$.
4. The next morning, remove any residual virus particles by replacing the virus-containing medium with fresh fibroblast medium (*see Note 23*).
5. Twenty-four hours post transduction, reseed the transduced cells in the fibroblast growth media from one well of a 6-well plate into one Matrigel-coated 60-mm dish.
6. The next day, replace the fibroblast medium with the reprogramming E7 medium. Observe the reprogramming fibroblasts with fluorescent microscope and make sure that the cells are expressing GFP (Fig. 3a).
7. Refresh the E7 medium daily. Small clusters with epithelial morphology should be seen at around day 10 of reprogramming (Fig. 3b).
8. From day 12–14, start to use the E8 medium for continued reprogramming, as well as expansion and proliferation of the reprogrammed iPSCs.
9. On day 21–25 of reprogramming, iPSC colonies can be picked up for establishment of cell lines, or can be directly stained for analyzing the expression of pluripotency markers such as alkaline phosphatase or TRA-1-60. For establishment of iPSC lines, pick up colonies with low or negative-GFP signals (Fig. 3c) and place one picked colony into one Matrigel-coated well of a 12-well or a 6-well plate (*see Subheading 2.5* for preparation of Matrigel-coated plates/dishes).
10. Expand the iPSCs that the GFP expression is progressively silenced using procedures described in Subheading 3.5 below (Fig. 3d) (*see Note 24*).

3.5 Culture Expansion and Freezing of the Established iPSCs

1. Refresh the E8 medium daily for the iPSCs established from one single colony at **steps 9 and 10** of Subheading 3.4, and culture iPSCs at 37 °C, 5% CO₂ until the neighboring colonies start to merge.
2. One hour before passaging, coat the plates with Matrigel and incubate the plates in a 37 °C incubator (*see Subheading 2.5* for preparation of Matrigel-coated plates).

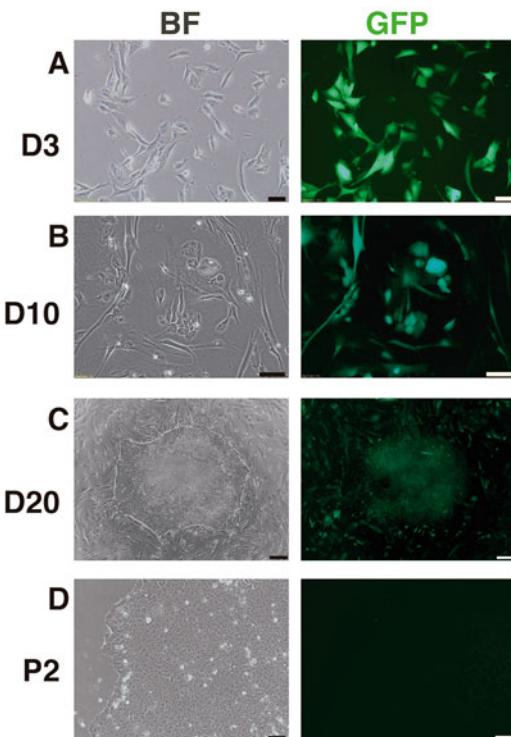


Fig. 3 Changes in morphology during reprogramming, and efficient silencing of transgenes in the established iPSCs. **(a, b)** Morphology of reprogramming fibroblasts on day 3 (**a**) and day 10 (**b**) (left panels), and transgene expression in the reprogramming fibroblasts on day 3 (**a**) and day 10 (**b**) as indicated by GFP expression (right panels). **(c)** A representative human iPSC colony (left panel) in the reprogramming vessel on day 20, and faint GFP expression (right panel) at this point indicating significant silencing in the iPS cells. **(d)** A representative iPSC colony at passage 2 showing complete silencing of transgenes in the established iPSC line (right panel). Scale bars, 50 μ M in (**a**, **b** and **d**); 200 μ M in (**c**). D3, day 3; P2, passage 2

3. Warm up E8 medium and the EDTA iPSC-dissociation solution to room temperature before passaging.
4. Remove and discard the spent E8 medium.
5. Add 1 mL of EDTA-dissociation solution into one well of a 6-well plate (0.5 mL for one well of a 12-well plate) and incubate in a 37 °C incubator for 3 min.
6. Add 1 mL per well of E8 medium for neutralization (0.5 mL for one well of a 12-well plate). Wash colonies off the plate by pipetting the medium.
7. Centrifuge the cells at $300 \times g$ for 5 min and remove the supernatant medium by vacuum aspiration.

8. Plate the appropriate amount of cells in E8 media into the freshly prepared Matrigel-coated wells/dishes (*see Note 25*). To promote attachment of iPSCs to the vessel, ROCK inhibitor Y-27632 should be used at a final concentration of 10 µM (*see Note 26*).
9. Early the next morning, replace the spent media with fresh E8 media to remove the ROCK inhibitors (*see Note 27*).
10. Culture iPSCs for 5–7 days with daily change of media until 80% of confluence.
11. For freezing the cells, after **step 7**, resuspend the cells in the freezing medium at a concentration of $0.5\text{--}1 \times 10^6$ cells/mL and transfer 1 mL of cells into one cryogenic vial (*see Note 28*). Place the vials with iPSCs in a cryogenic container and immediately transfer the freezing container into a -80°C freezer, and pre-freeze overnight.
12. The next day, transfer the iPS cells to liquid nitrogen for long-term storage (*see Note 29*).

4 Notes

1. The envelope and packaging plasmids are widely used. If these two plasmids are not available in the user's laboratory, plasmids pMD2.G and psPAX2 are available from Addgene with catalog numbers of 12259 and 12260, respectively.
2. These three reprogramming factors are used in this protocol because the co-expression of GFP provides a convenience for viral titration using flow cytometry, monitoring of transfection and transduction efficiency, as well as a marker for transgene silencing in the reprogrammed iPSCs. Other lentiviral reprogramming constructs available in Addgene or in the users' laboratory may be used.
3. Reprogramming can be carried out in an ordinary CO_2 tissue culture incubator. iPSC reprogramming can be enhanced in a hypoxia condition (5% oxygen) [12] if a hypoxia incubator is available.
4. FGF2 in the fibroblast growth medium is beneficial to reprogramming because it maintains a healthy state of fibroblasts and promotes the expression of OCT4 in fibroblasts even in the absence of reprogramming factors [13], but the FGF2 supplement is not essential for the maintenance of fibroblasts.
5. The osmolarity of E8 medium should be 340 mOsm and the pH is 7.4. Adjust the pH to 7.4 using 10 N NaOH. The amount of sodium bicarbonate are based on the specific DMEM/F-12 discussed below. The components of DMEM/F-12 vary in terms of HEPES, L-glutamine, and sodium

bicarbonate. We use the powder form with HEPES but without sodium bicarbonate (Thermo Fisher Scientific, Cat# 12400024). Use this DEME/F-12, we do not adjust the osmolarity and pH when we add the specified amount of sodium bicarbonate here. This is helpful when the osmometer is not available in the users' laboratory. The liquid form with HEPES can also be used (Thermo Fisher Scientific, Cat# 11330-032).

6. Vitamin C is not stable. This protocol uses the stabilized, synthetically derived version of vitamin C, L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate. Do not use the conventional vitamin C that might be available in the chemical stock of the users' laboratory. The molecular weights of these two versions are also different.
7. E8 medium can be prepared in large volume (e.g., 10 L) as base media without the unstable components. This base medium does not need sterilization by filtration if frozen immediately at -20 °C as 500-mL aliquots in used but clean plastic medium bottles. Do not use glass bottles for this purpose because glass bottles may crack during storage in very low temperature. The unstable components (FGF2, TGF beta, insulin, and transferrin) are added to prepare the fresh working complete E8 medium before use. The fresh complete E8 medium should be sterilized by filtration and stored at 4 °C. It is not encouraged to use the E8 media after 14 days of storage at 4 °C. To maintain potency of the E8 media, it is encouraged to use the genetically modified heat-stable FGF2. Antibiotics can be added, but are not necessary when cares are properly exerted. The E8 medium is commercially available at a higher cost.
8. Upon arrival, Matrigel stock should be aliquoted in a small volume, and stored at -80 °C. Avoid multiple rounds of thawing-freezing of Matrigel. Prepare Matrigel on ice with pipette tips and tubes that are pre-cooled at -20 °C. The diluted Matrigel should be used immediately. The coated plates/dishes should be used the same day. Coated plates/dishes should be stored at 4 °C if not used the same day. Coated plates/dishes stored at 4 °C should be warmed up at 37 °C for at least 30 min before use.
9. The collagen solution can be reused. Collect the coating collagen solution from the coated plates/dishes aseptically, and put it back into the collagen-solution bottle for reuse.
10. Your laboratory should have the approval to use lentiviral vectors. Extreme care should be observed when working with viral particles. Proper PPE should be used when handling lentiviral vectors. It is encouraged to wear double gloves when handling lentiviral particles. All laboratory materials that come into contact with viral preparations should be treated with bleach solution to kill the residual viruses.

11. Endotoxin-free Maxiprep kits are preferred but are not essential. Because of instability of the transfer plasmids, it is encouraged that the *E. coli* strain Stbl3, which reduces the chance of homologous recombination of the resident plasmids, should be used as the host strain of the lentiviral plasmids.
12. Because of repetitive sequences of the long terminal repeats (LTR), the transfer plasmids tend to lose one LTR. There is one AflII site in each of the two LTRs, and a third AflII site within the EF1 α promoter sequence. A diagnostic restriction with AflII can quickly examine the integrity of the plasmids (Fig. 1b).
13. Multiple dishes can be transfected simultaneously to prepare a large batch of viruses. Scale up the DNA/calcium based on number of dishes for virus packaging.
14. For convenience of volume calculation, PBS buffer can be used to adjust the total volume of the precipitation preparation. Our experience is that the concentration of NaCl can vary within 0.3–0.4 M without negative effect on virus yields. But, the concentration of PEG-6000 should be 8.5% for good results.
15. To pellet the viral particles, centrifuge the virus-containing liquid for 10 min at $7,000 \times g$ is recommended. But, many laboratories do not have a centrifuge that can run a rotor for large tubes (50- and 500-mL tubes) at $7,000 \times g$. We found that longer centrifugation at lower force can pellet the viruses. At $3,900 \times g$, 30 min to 1 h of centrifugation works well. After centrifugation, a white pellet should be visible. Always keep the tubes with the viruses on ice after this step.
16. Remove PEG-NaCl solution as completely as possible with a Pasteur pipet attached to a vacuum line. Most of the PEG-NaCl solution leftovers or drops stay on the wall of the tubes; therefore, it is recommended to roll the pipette tip around the wall to suck out the residual PEG-NaCl liquids. But, do not let the sucking pipette to touch the viral pellet. It is advised to keep the viral pellet in TBS buffer on ice for 1 h to promote resuspension of viruses.
17. Avoid high temperature during this procedure. Keep the master tube containing the resuspended viruses on ice during the entire aliquoting process. The viral aliquots should be snap-frozen immediately after each aliquot. To shorten the time for aliquoting, two lab mates can work together.
18. Use fresh pipette tips to distribute the different viruses when titrating different reprogramming factors. Treat the unused viruses with bleach solutions.
19. A dilution factor of 100 is generally appropriate for titers of around 10^8 TU/mL.

20. Our experience is that values below 20% of GFP⁺ cells fall into the linear stage of the titration curve, in which it is less likely that there are more than one viral integrations per cell. Values below 1% may not predict the titers reliably. Therefore, we do not include values less than 2% or more than 20% in our calculation of viral titers.
21. For best results, use human fibroblasts at a low passage number as the starting cells for reprogramming. This protocol has been tested using fibroblasts with a passage number below 10.
22. The stoichiometry of the three reprogramming viruses is critical. Higher MOI for OCT4 is needed. We empirically use an MOI ratio of (8–10):5:5 for OCT4:KLF4:SOX2.
23. The spent media should be treated with bleach to kill any residual live viral particles. The sucking pipette tips used should also be decontaminated using bleach solution.
24. It may take several passages for the GFP to be completely silenced in iPSCs.
25. For the first EDTA passaging, the cells can be seeded into one well of a 6-well plate because there are not sufficient cells. For the established iPSC lines, the culture can be split every 5–7 days at 1:8–1:12 ratio.
26. In place of Y-27632, thiazovivin can be used at a final concentration of 1 μM [5].
27. ROCK inhibitors trigger differentiation of iPSCs, but iPSCs can recover if treatment of ROCK inhibitors is short. Do not include the ROCK inhibitors in the iPSC culture media for too long. To this end, passage iPSCs in the late afternoon before leaving the laboratory on the day of cell passage. The next day, the first thing to do is to remove the ROCK inhibitors by changing media.
28. With E8 system, the human iPSCs survival rate is much higher than that with the traditional feeder-based culture system. We usually freeze cells from one well of a 6-well plate into three vials. In the feeder culture system, cells from one well are frozen into one vial.
29. Do not store the cells at –80 °C for more than 24 h before transfer into liquid N₂.

Acknowledgments

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Chapter 8

Episomal Reprogramming of Human Peripheral Blood Mononuclear Cells into Pluripotency

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Abstract

Peripheral blood is an easily accessible cell resource for reprogramming into pluripotency by episomal vectors. Here, we describe an approach for efficient generation of integration-free induced pluripotent stem cells (iPSCs) under feeder or feeder-free conditions. Additionally, in combination with the CRISPR-Cas9 genome-editing system, we can directly generate edited iPSCs from blood cells. With this protocol, one can easily generate either integration-free iPSCs or genetically edited iPSCs from peripheral blood at high efficiency.

Key words Induced pluripotent stem cells, Peripheral blood, Episomal vectors, Reprogramming, Genomic editing

1 Introduction

By forced expression of Yamanaka factors (OCT4, SOX2, MYC, and KLF4), one can reprogram somatic cells into induced pluripotent stem cells (iPSCs) [1–5]. This advance has opened opportunities for personalized cell therapy, disease modeling, and drug screening [6–8]. Peripheral blood (PB) is widely used in medical diagnostics and is easily obtained. By gradient centrifugation, one can obtain peripheral blood mononuclear cells (PBMNCs) from fresh PB samples or existing stocks. For the derivation of clinical-grade iPSCs, reprogramming methods that minimize the chances of insertional mutagenesis are preferred. The Sendai virus vectors reprogram PBMNCs at high efficiency [9]. However, the Sendai virus method is less affordable. In contrast, episomal reprogramming is an ideal and cost-effective method for creating clinical-grade and integration-free iPSCs. Transfecting EBNA1/oriP-based episomal vectors expressing Yamanaka factors in PBMNCs by nucleofection can generate footprint-free iPSCs [10, 11]. We have optimized the episomal reprogramming

[12–14]. One can use our optimized protocol to obtain thousands of colonies from 1 mL of PB.

CRISPR-Cas9 is a powerful genome-editing technology [15–17] and has been widely used for editing iPSCs [18, 19]. Conventionally, iPSCs are generated from somatic cells first, and then the iPSC genome is edited [20–23]. To facilitate and simplify the reprogramming and editing processes, we have established simultaneous reprogramming and genome editing of PB by one nucleofection of episomal vectors for both reprogramming and CRISPR-Cas9 editing [24].

In this chapter, we describe a protocol for the highly efficient episomal reprogramming of PB under feeder-dependent or feeder-free conditions. In combination with the CRISPR-Cas9 system, we provide procedures for simultaneous reprogramming and gene-editing of PB to generate genetically edited iPSCs by a single nucleofection.

2 Materials

2.1 Cells

1. Human peripheral blood is obtained from anonymous adult donors with no patient identification from the local blood center.
2. Mouse embryonic fibroblasts (MEFs) are prepared inhouse from E13.5 mouse embryos.
3. Feeder cells are derived by culturing MEFs to passage 4 or 5. MEFs are inactivated by treating with 10 µg/mL Mitomycin C for 3 h.

2.2 MNC Isolation

1. Ficoll-Paque Premium (1.077 g/mL).
2. DPBS, no calcium, no magnesium.
3. (Optional) Red Blood Cell Lysis Buffer (RBC lysis buffer): Dissolve 8.3 g NH₄Cl and 1.0 g KHCO₃ in 900 mL of water, and add 1.8 mL of 5% EDTA. Adjust the pH to 7.2–7.4. Bring the volume to 1.0 L with water. Filter-sterilize through a 0.2 µm filter. Store at 4 °C, but warm up to room temperature before use.

2.3 Cell Culture

1. Erythroid culture medium: Stemline® II Hematopoietic Stem Cell Expansion Medium supplemented with 100 ng/mL Recombinant Human SCF, 10 ng/mL Recombinant Human IL3, 2 U/mL Recombinant Human EPO, 20 ng/mL Recombinant Human IGF1, 1 µM dexamethasone, and 0.2 mM 1-thioglycerol (MTG). Filter-sterilize through a 0.2 µm filter (optional) and store the medium at 4 °C for up to 3 weeks.

2. Washing media: IMDM for PBMNCs; DMEM for MEF and feeders; Knockout DMEM/F12 medium for iPSCs.
3. MEF/feeder culture medium: DMEM (high glucose) medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin. Store at 4 °C for up to 4 weeks.
4. Mitomycin C (1 mg/mL): Dissolve 2 mg mitomycin C in 2 mL DPBS. Store at –20 °C for up to 6 months.
5. Sodium butyrate (0.25 M): Dissolve 1.1 g sodium butyrate in 40 mL of water (*see Note 1*). Store at –80 °C for at least 1 year.
6. iPSC generation medium: Mix Essential 8 (E8) basal medium and the provided medium supplements, supplemented with 0.25 mM sodium butyrate. Store at 4 °C for up to 4 weeks.
7. iPSC culture medium: Either E8 or StemFlex™ medium alone can be used for iPSC culture under feeder-free conditions. We prefer to use a medium containing 50% E8 and 50% StemFlex™ medium.
8. ROCK inhibitor Y-27632 (10 mM): Dissolve 10 mg Y27632 in 3 mL DMSO. Store at –80 °C for at least 1 year.
9. iPSC passage medium: iPSC culture medium supplemented with 10 µM ROCK inhibitor Y-27632 (*see Note 2*).
10. iPS Cell detachment solution: 0.5 mM EDTA in DPBS.
11. Matrigel (1:100 dilution): Add 1 mL of Matrigel to 99 mL of Knockout DMEM/F12 medium supplemented with 1% penicillin/streptomycin. Store at 4 °C for up to 1 month. Before passaging iPSC without feeder support, add 1 mL of Matrigel (1:100 dilution) into each well of the tissue culture (TC)-treated 6-well plates. Incubate at 37 °C for 1 h or 4 °C overnight. Aspirate the Matrigel and add medium and cells for culture.
12. Reprogramming attachment medium: DMEM (high glucose) medium supplemented with 2% fetal bovine serum (FBS) or 2% BSA, and 10 ng/mL BMP4. Store at 4 °C for up to 4 weeks.
13. Single-cell passage medium: StemFit medium (Ajinomoto; Cat. No. Basic02) supplemented with 50 ng/mL FGF2 and 10 µM ROCK inhibitor Y-27632.
14. Single-cell detachment solution: Accutase.

2.4 Freezing Medium

1. Freezing medium stock (2×): Dissolve 5 g trehalose in 30 mL water in a 37 °C water bath, bring the temperature to 4 °C and then add 10 mL of FBS and 10 mL of DMSO [25]. Filter-sterilize with a 0.22 µm syringe filter. Store at 4 °C for up to 3 months.

2. MEF/feeder freezing medium: Mix an equal volume of the freezing medium stock ($2\times$) and the MEF/feeder culture medium.
3. PBMNC freezing medium: Mix an equal volume of the freezing medium stock ($2\times$) and the erythroid culture medium.
4. iPSC freezing medium: Mix an equal volume of the freezing medium stock ($2\times$) and the iPSC culture medium and supplement with 10 μM ROCK inhibitor Y-27632.

2.5 Episomal Vectors for Reprogramming [14]

1. pEV-SFFV-OCT4-E2A-SOX2-Wpre (pEV-O/S).
2. pEV-SFFV-MYC-Wpre (pEV-M).
3. pEV-SFFV-KLF4-Wpre (pEV-K).
4. pEV-SFFV-BCL-XL-Wpre (pEV-B).

2.6 CRISPR-Cas9 Vectors for Genomic Editing [24]

1. pEV-SFFV-Cas9-E2A-KLF4-Wpre (pEV-Cas9/KLF4).
2. pEV-SFFV-SV40LT-Wpre (pEV-SV40LT).
3. pPU6-sgRNA vectors, such as pPU6-sgPRDM14 and pPU6-sgCTNNB1.
4. Donor vectors, such as pD-PRDM14 and pD-CTNNB1 (see Note 3).

2.7 Endotoxin-Free Plasmid Preparation

1. Use a commercial Plasmid Purification Maxiprep Kit to extract episomal vectors from *E. coli* according to the manufacturer's protocol. For the final step, substitute TE buffer with endotoxin-free sterile water to dissolve the DNA pellet.
2. Measure DNA concentration using a commercial UV/Vis spectrophotometer. The concentration is usually greater than 1 $\mu\text{g}/\mu\text{L}$, with the A260/A280 and A260/A230 ratios greater than 1.8 and 2.0, respectively.

2.8 Nucleofection Equipment and Kit

1. Nucleofector™ 2b (Lonza) or 4D-Nucleofector™ (Lonza, Core Unit, and X Unit).
2. For Nucleofector™ 2b, Amaxa® Human CD34+ Cell Nucleofector® Kit (Lonza, VPA-1003) is used.
3. For 4D-Nucleofector™, P3 Primary Cell 4D-Nucleofector™ X Kit (Lonza, V4XP-3024) is used.

2.9 Plastics and Equipment

1. Non-TC-treated 6-well plates.
2. TC-treated 6-well plates.
3. TC-treated 24-well plates.
4. 15- or 50-mL polystyrene tubes.
5. 10 mL syringes with a long needle.
6. Pipettes and pipettors.
7. Chamber slides.

8. CO₂ incubator.
9. Bucket centrifuge.
10. Hypoxia chamber.
11. Mixed gas cylinder. Order a mixed gas composed of 3% O₂, 5% CO₂, and 92% N₂. Flush the hypoxia chamber at 30 L/min for 1 min.
12. Flow cytometry cell analyzer.
13. Inverted microscope.
14. Confocal microscopy.

2.10 Primers

2.11 Immuno-histochemistry and Flow Cytometry

1. Flow cytometry (FACS) buffer: 2% FBS, 2 mM EDTA in DPBS.
2. Fixation buffer: 4% PFA in DPBS.
3. Permeabilization buffer: 0.1% Triton-X 100 in DPBS.
4. Blocking buffer: 5% goat serum in DPBS.
5. Antibodies: Anti-OCT4, Anti-NANOG, TRA-1-60-PE Antibody, eFluor 570-conjugated SSEA4, fluorophore-conjugated secondary antibody, and isotype antibody.
6. DAPI (10 mg/mL): Dissolve 1 mg DAPI in 1 mL ddH₂O.

3 Methods

Conduct all experiments at room temperature (22–25 °C) unless otherwise specified.

3.1 Isolation of Peripheral Blood Mononuclear Cells (PBMNCs)

1. Add 10 mL of DPBS to 10 mL of fresh peripheral blood (PB) in a 50 mL tube. Mix well with a 10 mL pipette (*see Note 4*).
2. Slowly add 10 mL of Ficoll-Paque (1.077 g/mL) to the bottom of the tube using an 18 G needle attached to a 10 mL syringe. This process should be done slowly to ensure that the Ficoll layer does not mix with the PB (*see Note 5*).
3. Centrifuge at 400 × *g* for 30 min at a low acceleration and deceleration rate.
4. After centrifugation, there will be several layers of different cell types, and the PBMNCs are in the white layer (buffy coat) located between the PB plasma and the Ficoll. Slowly aspirate ~10 mL PB plasma without disturbing the white layer. Place a 1 mL pipette tip at ~1 mm above the white layer and carefully harvest cells in the white layer into a new 50 mL tube. When the PBMNCs are collected sufficiently, the total volume will be about 3–6 mL.

5. Add 4–6 volumes (of collected PBMNCs in **step 4**) of DPBS to bring the total volume to 30 mL and mix well with a 10 mL pipette. If there are more than 30 mL, split it into two tubes (*see Note 6*).
6. Mix by pipetting to generate a single-cell suspension. Centrifuge the cells at $400 \times g$ for 10 min, and then remove the supernatant. Resuspend the cell pellet in 20 mL of the PBMNC washing medium and centrifuge at $400 \times g$ for 5 min to remove the majority of the platelets.
7. Resuspend the cells in 2 mL of erythroid culture medium or PBMNC freezing medium and count the number of cells. Cells may be used for immediate culture or frozen down for later use.
8. For cryopreservation, aliquot cells in cryovials (0.5–1 mL per vial) and transfer cryovials to a -80°C freezer immediately. PBMNCs may be stored in -80°C freezer for several days or transferred to a liquid nitrogen tank for long-term storage.

3.2 PBMNCs Culture in Erythroid Medium

1. Quickly thaw the frozen PBMNCs in a 37°C water bath and then transfer them to a 15 mL tube with 5 mL of the PBMNC washing medium.
2. Centrifuge the cells at $400 \times g$ for 5 min and then remove the supernatant. Resuspend the cell pellet in 5 mL of the PBMNC washing medium and centrifuge at $400 \times g$ for 5 min to remove residual DMSO.
3. Remove the supernatant and resuspend the cell pellet in the erythroid culture medium. Culture PBMNCs at $2\text{--}5 \times 10^6/\text{mL}$ (*see Note 7*). Add 2 mL to each well of a non-TC-treated 6-well plate. Add 1 mL of erythroid culture medium into each well every 2–3 days.
4. Culture the cells at 37°C for 6 days in a 5% CO_2 incubator with a tray of water to maintain the humidity.

3.3 Feeder Cell Preparation

For feeder-free reprogramming, skip this section and *see Subheading 3.4*.

1. One day before nucleofection, thaw mitomycin C-treated MEF cells (feeder cells) by adding 5 mL of the MEF washing medium. Approximately $1\text{--}3 \times 10^6$ feeder cells are seeded in 3 wells of a 6-well plate.
2. Spin down the cells at $200 \times g$ for 5 min. Resuspend the cells in 6 mL of the MEF/feeder culture medium, and add 2 mL of the cell suspension to each well of a tissue culture-treated 6-well plate that has been pre-coated with 0.1% gelatin at 37°C for 20 min.

3. Culture cells at 37 °C in a 5% CO₂ humidified incubator.
4. Before nucleofection, aspirate the MEF/feeder culture medium, and add 1–1.5 mL of erythroid culture medium.
Go to Subheading 3.5.

3.4 Feeder-Free Reprogramming

3.5 Nucleofection

3.5.1 For Nucleofector™ 2b

1. On the day of nucleofection, pre-coat 6-well plate with 1 mL of 0.1 mg/mL fibronectin (FN) in each well for at least 10 s (*see Note 8*).
2. Before nucleofection, aspirate the FN and add 1 mL of erythroid culture medium immediately.
1. For reprogramming alone without genome editing, add 2 µg pEV-O/S, 1 µg pEV-M, 1 µg pEV-K, and 0.5 µg pEV-B in a sterile Eppendorf tube. For simultaneous reprogramming and genome editing, add 2 µg pEV-O/S, 1 µg pEV-M, 0.5 µg pEV-B, 4 µg pEV-Cas9/KLF4, 1 µg pEV-SV40LT, 2 µg Donor Vectors (such as pD-PRDM14), and 1 µg pPU6-sgRNA Vectors (such as pPU6-sgPRDM14) in a sterile Eppendorf tube.
2. Heat the tube at 50 °C for 5 min to prevent contamination. Cool down the plasmid mixtures at room temperature for 5 min. Add 57 µL of nucleofection buffer and 13 µL of the supplement provided in the Human CD34⁺ Cell Nucleofector® Kit. Mix the DNA and the buffer with a 100 µL tip.
3. Count the number of cells and harvest 2 × 10⁶ cells to 15 mL tube. Spin down the cells at 400 × g for 5 min. Carefully aspirate the medium before adding the nucleofection buffer (*see Note 9*).
4. Add the DNA and nucleofection buffer mix to the cell pellet. Mix well by flicking the tube with your finger.
5. Transfer the DNA and cell suspension mix to the cuvette and cap the cuvette. Select the Program U-008 on the nucleofection device. Insert the cuvette into the holder and press OK to run the U-008 program (*see Note 10*).
6. After nucleofection, add 0.5 mL pre-warmed erythroid culture medium in each cuvette and transfer the cells to the 6-well plates with feeder cells or into FN pre-coated plates immediately. Due to the high reprogramming efficiency and donor variation, seeding different numbers of cells ranging from 1–10 × 10⁵ cells per well is highly encouraged. Add 1.5 mL erythroid culture medium to each well of a 6-well plate.
7. Transfer the plates into a hypoxia chamber, flush with a mixed gas composed of 3% O₂, 5% CO₂, and 92% N₂, and culture the cells at 37 °C.

3.5.2 For 4D-Nucleofector™

- For reprogramming alone without genome editing, add 2 µg pEV-O/S, 1 µg pEV-M, 1 µg pEV-K, and 0.5 µg pEV-B in a sterile Eppendorf tube. For simultaneous reprogramming and genome editing, add 2 µg pEV-O/S, 1 µg pEV-M, 0.5 µg pEV-B, 4 µg pEV-Cas9/KLF4, 1 µg pEV-SV40LT, 2 µg Donor vectors (such as pD-PRDM14) and 1 µg pPU6-sgRNA vectors (such as pPU6-sgPRDM14) in a sterile Eppendorf tube.
- Heat the tube at 50 °C for 5 min to prevent contamination. Cool the plasmid mixtures at room temperature for 5 min. Add 82 µL of nucleofection buffer and 18 µL of the supplement provided in the P3 Primary Cell 4D-Nucleofector™ X Kit. Mix the DNA and the buffer with a 100 µL pipette tip.
- Count the number of cells and harvest 2×10^6 cells to a 15 mL tube. Spin down the cells at $400 \times g$ for 5 min. Carefully aspirate the medium before adding the nucleofection buffer.
- Add the DNA and nucleofection buffer mix to the cell pellet. Mix well by flicking the tube with your finger.
- Transfer the DNA and cell suspension mix to the cuvette and cap the cuvette. Select the buffer P3 and Program EN-138 on the nucleofection device. Press OK and insert the cuvette into the holder and press start to run the EN-138 program (*see Note 11*).
- After nucleofection, add 0.5 mL pre-warmed erythroid culture medium in each cuvette and transfer the cells onto the plates with feeder cells or onto the FN-coated plate immediately. Due to the high reprogramming efficiency and donor variation, seeding different numbers of cells ranging from $1-10 \times 10^5$ cells per well is highly encouraged. Add 1.5 mL erythroid culture medium into each well of a 6-well plate.
- Transfer the plate to a hypoxia chamber, flush with a mixed gas composed of 3% O₂, 5% CO₂, and 92% N₂, and culture the cells at 37 °C.

3.6 iPSC Generation (Feeder-Dependent)

- On day 2 post-nucleofection, directly add 1.5 mL of iPSC generation medium into each well. Do not aspirate out the erythroid culture medium.
- Starting on day 4, change the medium every 2 days, leaving 0.5 mL spent medium (*see Note 12*) and adding 2 mL of iPSCs generation medium to the well. Most of the live cells will have attached to the feeder layer on day 4 post-nucleofection (*see Note 13*).
- Small iPSC-like colonies often appear at 1 week after nucleofection (*see Note 14*).

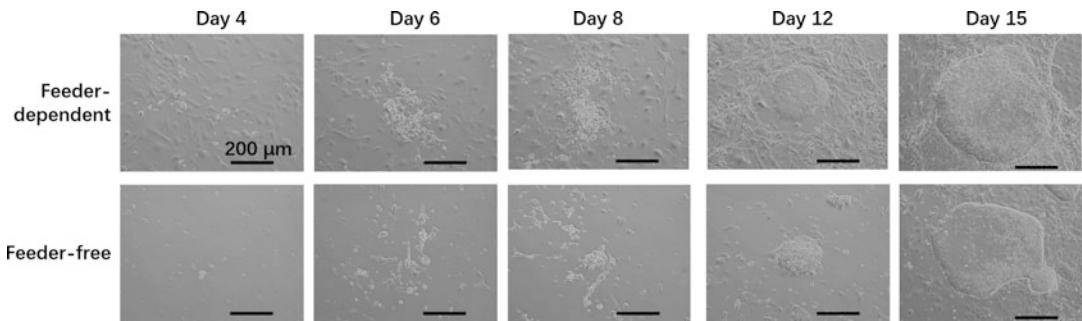


Fig. 1 Cell morphology during episomal reprogramming under feeder-dependent and feeder-free conditions. Scale bar: 200 μm

4. On day 12–18 post-nucleofection, feed iPSC culture medium when iPSC-like colonies grow larger (Fig. 1).

3.7 iPSC Generation (Feeder-Free)

1. On day 2 post-nucleofection, directly add 1.5 mL of reprogramming attachment medium into each well [26]. Do not aspirate out the old erythroid culture medium.
2. On day 3 post-nucleofection, aspirate out 1.5–2 mL culture medium and leave 1 mL culture medium in each well. Do not disturb cells. Add 1.0 mL of iPSC generation medium into each well.
3. Starting on day 4 follow protocols described in Subheading 3.6.

3.8 Picking iPSC Colonies and Long-Term Feeder-Free Culture

1. Before picking colonies, prepare the Matrigel-coated 24-well plate (*see Note 15*). To establish multiple iPSC lines, we recommend picking at least 10 colonies for each donor.
2. Add 500 μL iPSCs passage medium in each well of feeder or Matrigel-coated 24-well plate. Warm the medium at 37 °C in a 5% CO₂ humidified incubator for 15 min.
3. Use a 10 or 20 μL pipette to scratch and pick colonies that are large and well separated from others under the microscope. Transfer the pieces of each colony into the wells containing the pre-warmed culture medium (*see Note 16*).
4. Do not change medium for the first 2 days. ROCK inhibitor can promote the survival of small iPSC colonies.
5. From day 2 onwards, change iPSC culture medium every 2 days (*see Note 17*).
6. After approximately 1-week's culture, the colonies are large enough for passage. Remove the medium and add 200 μL cell detachment solution at 37 °C for 1–3 min. Remove the cell detachment solution and resuspend iPSCs in 500 μL iPSC

passage medium. Break down the colonies into clumps of 5–50 cells. Do not break down colonies into single cells, which may lead to excessive cell death.

7. Transfer cell suspension into each well of the Matrigel-coated 12-well plate or 6-well plate and mix well immediately. Culture cells in iPSC passage medium at 37 °C in a 5% CO₂ humidified incubator.
8. Change iPSC culture medium on the second day after passage, and every 2 days thereafter (*see Note 17*).
9. When the cells reach 60–80% confluence, treat the cells with 1 mL cell detachment solution at 37 °C for 1–3 min. When the colonies start to curl up, remove the cell detachment solution and add iPSCs passage medium to suspend the iPSCs. Passage cells with a splitting factor of 4–8. ROCK inhibitor should be added when passaging cells to increase survival and removed 1 day later to prevent differentiation.

3.9 Freezing iPSCs for Long-Term Storage

1. When the cells reach 50–70% confluence, aspirate the spent medium and treat the cells with 1 mL cell detachment solution at 37 °C for 1–3 min.
2. When the colonies start to curl up, remove the cell detachment solution and add iPSC freezing medium to resuspend iPSCs.
3. Mix well and transfer the vial to a –80 °C freezer. Frozen iPSCs can be stored in a –80 °C freezer for short-term storage (several days). For long-term storage, the vials may be transferred to a liquid nitrogen tank when the cells are frozen.

3.10 Thawing iPSCs

1. Quickly thaw the frozen iPSCs in a 37 °C water bath and then transfer them to a 15 mL tube with 5 mL of the iPSC washing medium.
2. Centrifuge the cells at 200 × *g* for 5 min and remove the supernatant. Resuspend the cell pellet in 5 mL washing medium and centrifuge at 200 × *g* for 5 min to remove the residual DMSO.
3. Aspirate out the supernatant and resuspend the cells in 2 mL iPSC passage medium. Culture the cells in a Matrigel-pre-coated 6-well TC-plate (*see Note 18*).
4. Starting the next day, refresh the cells with 2 mL iPSCs culture medium. Long-term culture and passage the iPSCs as described above.

3.11 Generation of Single-Cell Derived iPSCs Clones (Optional)

1. When passage iPSCs from one well of a 6-well TC-plate, aspirate the spent medium and treat the cells with 1 mL single-cell detachment solution at 37 °C for 3–5 min to generate a single-cell suspension.

2. Add 1/100 or less (<20 µL) into one well of a Matrigel-coated 6-well TC-plate with 2 mL of single-cell passage medium (*see Note 19*).
3. Several hours later, mark the single cells using a Nikon object marker.
4. When these single cells become large round colonies, start to pick and passage them as described above (*see Note 20*).

3.12 Selection of iPSCs Colonies without Residual Episomal Plasmids

1. After passage 5, harvest iPSCs and extract genomic DNA using a Genomic DNA Extraction Kit for analysis of residual plasmid DNA in the cells by real-time PCR.
2. Negative control: Extract a negative control DNA from untransfected PBMNCs.
3. One copy per genome-positive control: Add 1.6 pg pEV-O/S plasmid into 1 µg negative control DNA.
4. Run qPCR to obtain a C_T mean ($C_T(\text{PRIMER of SAMPLE})$) for each primer (EBNA1, WPRE, and GAPDH) and sample.
5. qPCR is performed using a FastStart Universal SYBR Green Master on Step One 7500. The qPCR program is 50 °C for 2 min (holding stage); 95 °C for 10 min (holding stage); 40 cycles of 95 °C for 15 s and 60 °C for 1 min (cycling stage); 95 °C for 15 s, 60 °C for 1 min, 95 °C for 30 s, 60 °C for 15 s (melt curve stage).
6. Calculate residual episomal plasmids copy number in iPSC using the following formula:

$$\begin{aligned} \text{Copy number } [(EBNA1 \text{ or } WPRE)] \\ = 0.5 CT(EBNA1 \text{ or } WPRE \text{ of iPSC}) - CT(GAPDH \text{ of iPSC}) \\ - CT(EBNA1 \text{ or } WPRE \text{ of one copy per genome positive control}) \\ + CT(GAPDH \text{ of one copy per genome positive control})] \end{aligned}$$

3.13 Selection of Gene-Edited iPSC Clones

7. In most of the clones, the residual episomal plasmids are 0.001 copies or undetectable. Choose these clones for continued culture, and you may toss away the clones with more than 0.01 copies per cell.
1. Harvest iPSC colonies or single-cell derived clones and extract genomic DNA for analysis of genomic editing by PCR and Sanger sequencing.
2. Design specific primers to amplify CRISPR-Cas9 targeted genome sequence (*see Note 21*).
3. DNA is amplified with KAPA HiFi DNA polymerase. The PCR cycling conditions are 98 °C for 2 min, followed by 98 °C for

10 s, 66 °C for 15 s, 72 °C for 1 min/kb, for 35 cycles. PCR products are visualized by 1% agarose gel electrophoresis.

- Validate if colonies/clones are correctly edited by Sanger sequencing of the PCR products (*see Note 22*).

3.14 Off-Target Analysis

- Predict the top 15 putative off-target sites using a Web-based program (<http://www.rgenome.net/cas-offinder/>). Design primers to amplify these putative off-target sites using Primer3Plus.
- DNA is amplified, and high-throughput sequencing (HTS) of PCR products is performed using Hiseq-PE (paired-end) 150.
- Data are analyzed using the Web-based program Galaxy (<https://usegalaxy.org/>) or CRISPResso2 [27].

3.15 Verification of Pluripotency Surface Markers by Flow Cytometry

- For staining of cell surface markers TRA-1-60 and SSEA4, harvest iPSCs by treating with 0.5–1.0 mL single-cell detachment solution at 37 °C for 3–5 min to obtain a single-cell suspension. Use untransfected PBMNCs as a negative control.
- Spin down the iPSCs at 200 × g for 5 min. Remove the supernatant and resuspend the cell pellet in 0.5 mL FACS buffer.
- Directly add 5 μL PE-conjugated TRA-1-60, or 5 μL eFluor 570-conjugated SSEA4 or isotype antibody in 100 μL of cell suspension at room temperature for 20 min.
- After washing with 2 mL FACS buffer, resuspend the cells in 300 μL of FACS buffer. Conduct flow cytometric analysis using a cell analyzer. Ten thousand events are collected for each sample.

3.16 Confocal Examination of Pluripotent Transcription Factors

- Seed iPSCs in feeder coated chamber slides and culture for 4–5 days.
- Remove the culture medium and fix the cells with a fixation buffer at room temperature for 30 min.
- Treat the cells with permeabilization buffer at room temperature for 30 min. Wash the cells twice with DPBS.
- Block the cells with blocking buffer at room temperature for 1 h.
- During the blocking step, dilute the primary antibody Anti-OCT4 or Anti-NANOG in the blocking buffer.
- Incubate cells with the diluted antibody at 4 °C overnight.
- Wash twice with permeabilization buffer for 15 min and wash twice with DPBS for 15 min each.
- Incubate cells with the diluted fluorophore-conjugated secondary antibody at room temperature for 2 h.

9. Wash the cells twice with permeabilization buffer for 15 min, and further wash with DPBS twice for 15 min each time.
10. Stain the nuclei with 1 µg/mL DAPI at room temperature for 10 min.
11. Wash cells with DPBS twice for 15 min each time.
12. Capture images with a confocal microscope.

3.17 Digital Karyotyping (See Note 23)

1. Genomic DNA samples from indicated iPSCs are hybridized to HumanCoreExome arrays and stained and scanned using the Illumina HiScan system per standard protocol.
2. Log R ratio (LRR) is the logged ratio of observed probe intensity to expected intensity, any deviations from zero in this metric indicate copy number changes.
3. B allele frequency (BAF) is the proportion of the hybridized sample that carries the B allele as designated by the Infinium assay. A normal sample would have discrete BAFs of 0.0, 0.5, and 1.0 for each locus (representing AA, AB, and BB).
4. Choose the clones with normal karyotyping for culture and other studies.

3.18 Pluripotency Test by Teratoma Formation

1. Diluted Matrigel: mix 50% high concentration Matrigel and 50% Knockout DMEM/F12 on ice.
2. Harvest 1–2 × 10⁶ iPSCs using a cell scraper and resuspend cells in 200 µL diluted Matrigel.
3. Subcutaneously inject cells into the rear haunch of NOD/S-CID immunodeficient mice. Mice are maintained at pathogen-free animal facilities.
4. At 8–12 weeks after iPSC injection, dissect and fix teratomas in 10% formalin. After micro-sectioning, samples are stained with hematoxylin and eosin (H&E) and analyzed by a pathologist.

4 Notes

1. Sodium butyrate can increase reprogramming efficiency, likely by multiple mechanisms: (a) increase expression levels of reprogramming factors, (b) open up chromatin, thus allowing for effective access by reprogramming factors.
2. The use of ROCK inhibitor before cell freezing and right after cell thawing is critical for increased survival of iPSCs.
3. The pU6-sgRNA vectors and donor template vectors should be individually designed for different loci. The targeting efficiency may be variable for different loci.

4. Approximately 1×10^7 mononuclear cells can be obtained from 10 mL of fresh PB. After 6 days of culture, $3-5 \times 10^6$ cells will remain alive due to the death of mature cells during culture.
5. Keep Ficoll-Paque at room temperature. If stored at 4 °C, warm it up to room temperature before use.
6. If there is a red blood cell contamination at the buffy coat layer, add 4–6 volume of room temperature RBC lysis buffer instead of DPBS, and the total volume should be less than 30 mL. Centrifuge at $400 \times g$ for 10 min and remove the supernatant. Resuspend the cell pellet in 30 mL of DPBS.
7. Hematopoietic cells appear to proliferate better when they are cultured at higher densities. The cell number will decrease during the culture due to the death of mature cells.
8. One may use vitronectin instead of FN for feeder-free reprogramming [28].
9. As low as 2×10^5 cells may be used for nucleofection, but less iPSC colonies should be expected.
10. The Nucleofection program is critical for successful reprogramming. One may try other programs, such as U-014. But the reprogramming efficiency needs to be validated.
11. Similar to Nucleofector™ 2b, one can also test other programs when using 4D-Nucleofector™.
12. When changing the culture medium, leave 500 µL per well to prevent the drying out of these cells in the wells. This can also increase efficiency, in particular, for large-scale cell culture.
13. Additional feeder cells may be added in the culture wells whenever the majority of feeder cells have been detached. After thawing feeder cells, resuspend the cell pellet in a small volume of iPSCs generation medium (i.e., ~0.2 mL for one well of a 6-well plate) and then add feeder cells into the culture wells. Approximately 2% of FBS may be added to increase cell attachment. Alternatively, MEF- or feeder cell-conditioned media can also be used, but it is more laborious.
14. The reprogramming efficiency of PB from different donors is highly variable.
15. The concentration of Matrigel differs from one batch to another. Usually, we use a 1:100 dilution of Matrigel.
16. Transfer cells to a 5% CO₂ humidified incubator as soon as possible to increase cell viability. If you will pick more than six colonies, or it takes more than 15 min, seed cells in two or more 24-well plates during reprogramming, which allows for alternation.
17. When using E8 only, change the medium every day.

Table 1
Primers for PCR

Target	Sequence
EBNA1-F	TTTAATACGATTGAGGGCGTCT
EBNA1-R	GGTTTTGAAGGATGCGATTAAG
WPRE-F	GGTTAACGCGTCGACAAT
WPRE-R	GTTGCGTCAGCAAACACAGT
GAPDH-F	GAGTCCACTGGCGTCTTC
GAPDH-R	GACTGTGGTCATGAGTCCTTC
PRDM14-F	GACCAGGAGTGCTCTATGGC
PRDM14-R	AGGAAATAGAGAGAATCCGAATCTC

Primer pairs EBNA1-F&R and WPRE-F&R are used to assess the copies of the episomal vectors. Primers GAPDH-F&R are used to amplify the human GAPDH gene, which serves as a positive control for PCR. The primers PRDM14-F&R target sequences outside of the donor vectors (pD-PRDM14) template.

18. In case that the cell survival is poor, the use of feeders will increase cell survival.
19. Make sure most iPSCs in single-cell detachment solution are single cells. Seed density should be much lower than conventional cell passage to obtain more single-cell clones.
20. Although single-cell derived iPSCs can be conducted by single-cell sorting using a cell sorter, we recommend avoiding cell sorting due to the reduced cell viability after sorting.
21. The donor vector of pD-PRDM14 is to insert an E2A-GFP into the stop codon of PRMD14. One can design specific donors for deletion, insertion, point mutation, and correction. The primers (Table 1) target sequences outside of the donor vectors (pD-PRDM14) template, thus can only amplify the wild-type and successfully edited genomic sequences.
22. For different editing loci, the method of selection of genomic-editing iPSCs may differ.
23. Digital karyotyping can identify the deletion or duplication of small pieces of DNA. Although pEV-SV40LT may increase editing efficiency in some loci during simultaneous reprogramming and genome editing, one may exclude pEV-SV40LT because we identified an alteration on chromosome 5 in one gene-edited iPSCs clone using pEV-SV40LT [24].

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Chapter 9

Generation of Human iPSCs by Episomal Reprogramming of Skin Fibroblasts and Peripheral Blood Mononuclear Cells

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Abstract

Human-induced pluripotent stem cells (iPSCs) can be generated from patient-specific somatic cells by forced expression of the transcription factors OCT4, SOX2, KLF4, and c-MYC. Sustained expression of the transgenes during reprogramming is crucial for the successful derivation of iPSCs. Integrating retroviruses have been used to achieve the required prolonged expression; however, issues of undesirable transgene expression in the iPSC-derived cell types post reprogramming can occur. Alternative non-integrating approaches to reprogram somatic cells into pluripotency have been established. Here, we describe a detailed method for generating human iPSCs from fibroblasts and peripheral blood mononuclear cells (PBMCs) using the non-integrating episomal plasmids. The delivery of the episomal plasmids into the somatic cells is achieved using a nucleofection technique, and reprogramming is performed in chemically defined media. This process takes approximately 30 days to establish the iPSC colonies. We also describe a method for growing iPSCs on vitronectin as well as procedures for the long-term expansion of iPSCs on human fibroblast feeder cells.

Key words Induced pluripotent stem cells, iPSC, Episomal reprogramming, Human peripheral mononuclear cells, Stem cells

1 Introduction

Patient-specific induced pluripotent stem cells (iPSCs) offer a new avenue to study molecular mechanisms underlying inherited diseases. iPSCs were first generated using retroviral vectors to ectopically express four transcription factors Oct4, Sox2, Klf4 and c-Myc [1, 2]. Retroviruses can readily infect a wide range of cells; however, the major drawback of retroviruses is that they integrate into the genomes to be reprogrammed and pose a risk of insertional mutagenesis. The integrated reprogramming factors may also have residual expression in the established iPSCs, and thereby result in a distorted transcriptional profile of the iPSCs and alter their differentiation potential. These integrated transgenes may also reactivate in the iPSC-derived cells, which may result in tumor formation

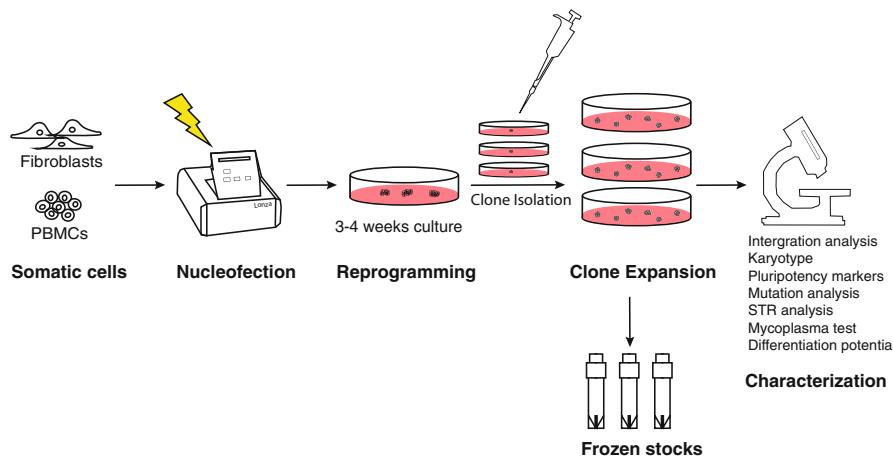


Fig. 1 Episomal reprogramming of human fibroblasts or PBMCs. Fibroblasts are expanded, or PBMCs are isolated from a patient and prepared for reprogramming. Nucleofection is used to transfet the episomal reprogramming plasmids into cells. Transfected cells are cultured for 3–4 weeks in E7 medium until iPSC colonies emerge. Colonies are isolated and expanded on vitronectin for freezing and further characterization

when transplanted into patients [3–5]. Since the initial iPSCs were described, an assortment of alternative approaches to generate transgene-free iPSCs have been developed, which include excisable Piggybac plasmids or non-integrating methods such as Sendai viruses, episomal plasmids, cell-permeable proteins and synthetic modified mRNA [6–10]. All of these approaches can generate transgene-free iPSCs at varying success rates.

In this chapter, we describe an episomal reprogramming method that can successfully reprogram patient fibroblasts and peripheral blood mononuclear cells (PBMCs) (Fig. 1). Episomal reprogramming is well established and cost-effective. In contrast to regular plasmids, episomal plasmids can remain in a cell and express their transgenes for a long period of time due to their ability to self-replicate and partition along cell division. Self-replication is achieved through the expression of the Epstein–Barr nuclear antigen-1 (EBNA1) and inclusion of the cis-acting oriP element on the plasmids [11].

The protocol described here omits a passaging step during the reprogramming phase used by many iPSC protocols, which reduces the possibility to produce many sister colonies from the same reprogramming event. This strategy helps to ensure that clones selected for characterization are from a unique reprogramming event and reduces the chances of selecting abnormal clones, which have a growth advantage [12]. We also describe how to expand iPSCs on vitronectin and provide procedures for long-term maintenance of the established iPSCs on human fibroblast feeder cells (Fig. 1).

2 Materials

All cell culture media and reagents should be prepared and used under aseptic conditions.

2.1 Plasticware and Other Consumables

1. 10 cm non-treated dishes (surface area 56.7 cm²).
2. T25 flasks (surface area 25 cm²).
3. T75 flasks (surface area 75 cm²).
4. Organ-culture dish, with a center well (surface area 2.89 cm²) (*see Note 1*).
5. 35 mm non-treated dishes (surface area 8.8 cm²).
6. Eppendorf tubes, 1.5 mL.
7. 25G × 5/8-in. (0.5 × 16 mm) needles.
8. Syringes (1 mL).
9. SepMate-50 tubes.
10. Lymphoprep (Density gradient medium).
11. 15 mL tubes.
12. 50 mL tubes.
13. 1.5 mL cryovials.

2.2 Reagents

1. Phosphate-buffered saline pH 7.4 (without calcium and magnesium; PBS−/−).
2. Phosphate-buffered saline pH 7.4 (with calcium and magnesium; PBS+/+).
3. DMEM/F12 media.
4. RPMI 1640 media.
5. Fetal calf serum (FCS) (*see Note 2*).
6. MEM Non-Essential Amino Acids Solution (100×): Glycine, L-Alanine, L-Asparagine, L-Aspartic acid, L-Glutamic Acid, L-Proline, L-Serine (all at 10 mM).
7. GlutaMAX (100×): 200 mM L-alanyl-L-glutamine dipeptide in 0.85% NaCl.
8. Dimethyl sulfoxide (DMSO).
9. TrypLE Express.
10. Trypan blue.
11. KnockOut Serum Replacement (KSR).
12. 2-Mercaptoethanol 50 mM (*see Note 3*).
13. Gelatin Type A from porcine skin.
14. Vitronectin XF (250 µg/mL).
15. Gentle Cell Dissociation Reagent.

16. Pen/Strep: penicillin-streptomycin solution with 10,000 U/mL penicillin and 10,000 µg/mL streptomycin.
17. P2 Primary Cell 4D-Nucleofector X Kit, Single Nucleocuvette (100 µL; Lonza, Cat# V4XP-2012).
18. P3 Primary Cell 4D-Nucleofector X Kit, Single Nucleocuvette (100 µL; Lonza, Cat# V4XP-3012).

2.3 Episomal Plasmids

1. pCXLE-hOct3/4-shp53-F (contains human *POU5F1* and an shRNA against *P53*; Addgene, Cat# 27077) (*see Note 4*).
2. pCXLE-hSK (contains human *SOX2* and *KLF4*; Addgene, Cat# 27078).
3. pCXLE-hUL (contains human *L-MYC* and *LIN28A*; Addgene, Cat# 27080).
4. pCXLE-EGFP (contains enhanced green fluorescent protein EGFP; Addgene, Cat# 27082) (*see Note 5*).

2.4 Growth Factors

1. Activin A (Stock concentration 25 µg/mL) (*see Note 6*).
2. FGF2 (Stock concentration 50 µg/mL) (*see Note 6*).

2.5 Preparation of Culture Media and Solutions

1. RPMI medium: 1× RPMI 1640 Media is supplemented with 10% FCS, 2 mM GlutaMAX, and 0.5% Pen/Strep solution (*see Note 7*).
2. E7 medium: 480 mL of E7 basal media mixed with 20 mL of E7 supplement (*see Note 8*).
3. E8 medium: 480 mL of E8 basal media mixed with 20 mL of E8 supplement (*see Note 8*).
4. KSR-medium: DMEM/F12 is supplemented with 2 mM GlutaMAX, 20% KSR, 0.1 mM NEAA, 90 µM 2-Mercaptoethanol, and 0.5% Pen/Strep solution (*see Note 7*).
5. Blood cell dilution solution: PBS-2% FCS, add 200 µL of FCS into 9.8 mL of PBS-/-, and mix well.

2.6 Preparation of Coated Dishes

1. Vitronectin-coated dishes. Prepare the working vitronectin XF solution by adding 40 µL of vitronectin into 1 mL of PBS-/- (the final concentration is 10 µg/mL). Coat a non-treated 35 mm dish with 1 mL of the diluted vitronectin solution and coat a non-treated 10-cm dish with 6 mL of the diluted vitronectin solution. Dishes should be coated for a minimum of 1 h at room temperature. Immediately before use, aspirate the vitronectin solution and wash the dish with PBS-/- one time, and then the vitronectin-coated dishes can be used to plate cells (*see Note 9*).
2. Gelatin-coated dishes. Prepare a gelatin stock solution of 1% by dissolving the gelatin in distilled water. Warm the solution to 37 °C and mix for approximately 1 h until it is completely

dissolved. Dilute the stock solution of gelatin in distilled water to make a 0.1% working solution and filter it immediately through a 0.22- μm filter. Coat the organ-culture dishes with 1 mL of 0.1% gelatin each and coat a T25 flask with 3 mL of gelatin solution. Leave at room temperature for 1 h and then aspirate the solution and allowed to dry for 1 h. Following the 1-h drying, plate cells onto the gelatin-coated dishes or flasks (*see Note 10*).

2.7 Human Fibroblasts for Use as Feeder Cells

Human neonatal fibroblasts CCD-1079Sk (ATCC; CRL-2097) (*see Note 11*).

2.8 Specific Equipment

1. Centrifuge with swing-out buckets that can hold 1.5 mL Eppendorf tubes.
2. 4D-Nucleofector system with Core and X-Unit (Lonza).
3. Stereomicroscope equipped with a transmitted-light base and dark-field.
4. Gamma Irradiator.

3 Methods

All experiments are performed under aseptic conditions in a Class II biological safety cabinet.

3.1 Preparation of Mitotically Inactivated Human Fibroblast Feeder Cells

1. Thaw the human fibroblasts CCD-1079Sk cells by holding the frozen cryovial (containing approximately 5×10^5 cells) in a 37 °C water bath until thawed.
2. Immediately and slowly dilute the 1 mL of cryopreserved cells with 9 mL of RPMI media (pre-warmed to 37 °C) in a 15-mL tube, and centrifuge at $125 \times g$ for 10 min at room temperature.
3. Remove the supernatant and gently resuspend the cells in 10 mL of fresh RPMI media (pre-warmed to 37 °C), supplemented with 20 ng/mL of FGF2.
4. Transfer the 10 mL of cell suspension to a T75 flask and add an additional 10 mL of RPMI media (pre-warmed to 37 °C), supplemented with 20 ng/mL of FGF2. Culture the cells at 37 °C and 5% CO₂ until they are 70% confluent and change media every 3 days.
5. When cells reach 70% confluence, aspirate the media and add 5 mL of PBS-/- to the cells. Remove the PBS-/- and add 5 mL of TrypLE Express. Incubate cells at room temperature for 5 min, tapping the flask occasionally. When most of the cells

have detached from the surface, add 5 mL of RPMI media to inactivate the enzymes and pipette the media across the surface of the flask several times to dislodge the remaining cells from the surface.

6. Transfer the 10 mL of cell suspension into a 15-mL tube and centrifuge at $200 \times g$ for 5 min.
7. Aspirate the media and resuspend the cell pellet in 10 mL of fresh RPMI media supplemented with 20 ng/mL of FGF2.
8. Dispense the cells at a ratio of 1:4 into four new T75 flasks in a total volume of 20 mL of RPMI media supplemented with 20 ng/mL of FGF2 per flask. Culture the cells at 37 °C and 5% CO₂, and change media every 3 days until cells are 70% to 90% confluent (approximately 1.5×10^6 to 2×10^6 cells per T75 flask) (*see Note 12*).
9. When cells reach 90% confluency, aspirate the media from all four flasks and add 5 mL of PBS—/— to each flask. Remove the PBS—/— and add 5 mL of TrypLE Express to each flask. Incubate cells at room temperature for 5 min, tapping the flasks occasionally. When most of the cells have detached from the surface, add 5 mL of RPMI media to each flask and pipette the media across the surface of the flasks several times to dislodge the remaining cells.
10. Transfer the 40 mL of cell suspension into a 50-mL tube and centrifuge at $200 \times g$ for 15 min.
11. Aspirate the media and resuspend the cell pellet (approximately 6×10^6 to 8×10^6 cells) in 20 mL of fresh RPMI media.
12. Place the 50 mL tube containing the cell suspension into the gamma irradiator and irradiate the fibroblasts to a dose of 50 Gray (*see Note 13*).
13. After irradiation, centrifuge the 50 mL tube at $200 \times g$ for 15 min at room temperature and resuspend the cells in ice-cold RPMI medium supplemented with 10% DMSO, to a concentration of 1×10^6 cells per mL. Dispense 1 mL of cell suspension per cryovial and place the cryovial into a freezing container at -80 °C overnight. The next day, transfer the cryovial into liquid nitrogen for long-term storage.
14. Prepare the feeder dishes 1 or 2 days before use. Specifically, thaw one vial of irradiated feeders by slowly diluting the 1 mL of cryopreserved cells with 9 mL of 37 °C RPMI medium.
15. Gently spin the cells at $125 \times g$ for 10 min and then resuspend the cells in 10 mL of 37 °C RPMI medium.
16. Add 1 mL (100,000 cells) to each gelatin-coated organ-culture dishes (surface area 2.89 cm²). Then, culture the cells overnight in an incubator at 37 °C and 5% CO₂ to allow cells to attach before use (*see Note 14*).

3.2 Expansion of Human Fibroblasts and Preparation of Cells for Nucleofection

1. Place 1 mL of RPMI media into the center of a 10-cm dish and allow it to spread out across an area of approximately 3 cm. Place the skin punch (approximately 4 mm in diameter) into the middle of the RPMI media. Using a pair of scalpel blades finely chop the skin punch into small fragments (approximately 10–15 fragments). Using a Pasteur pipette, transfer the skin fragments and the RPMI media into a gelatin-coated T25 flask and position the fragments evenly around the culture surface. Remove excess media around the fragments, so they are unable to float around, but leave the fragments sufficiently wet. Turn the flask upside down, so the skin fragments are at the top and add 5 mL of RPMI media into the flask while it remains inverted. Place the inverted flask in an incubator and culture overnight at 37 °C and 5% CO₂.
2. The following morning, carefully turn the flask right side up and allow the 5 mL of RPMI media to cover the skin fragments slowly. Check that the fragments are attached to the surface and are not floating.
3. Change media every 3 days and monitor the fragments for the outgrowth of fibroblasts (*see Note 15*).
4. After approximately 3–4 weeks, when the flask is confluent, passage the cells from the T25 flask into a T75 flask. Specifically, aspirate the media from the T25 flask and wash with 5 mL of PBS−/−. Aspirate the PBS−/− and add 3 mL of TrypLE Express. Incubate at room temperature for 5 min and tap the flask occasionally to help the cells detach from the culture surface. Pipette the cell suspension gently to help make a single-cell suspension and inactivate the TrypLE Express by diluting it with 5 mL of RPMI media.
5. Transfer the cell suspension to a 15-mL tube and centrifuge the cells at 200 × g for 10 min. Resuspend the cells in 10 mL of RPMI media supplemented with 20 ng/mL of FGF2.
6. Passage the cells into four new T75 flasks by transferring 2.5 mL of the cell suspension to each T75 flask and add to each flask 17.5 mL of RPMI media supplemented with 20 ng/mL of FGF2. Culture the cells at 37 °C and 5% CO₂, and change media every 3 days until they are 70% confluent (*see Note 16*).
7. Take one flask for nucleofection and remove media from the fibroblasts and wash cells twice with 5 mL of PBS−/− each time (e.g., 5 mL in a T75 flask).
8. Add sufficient TrypLE Express to the flask of fibroblasts to cover the culture surface (e.g., 5 mL for a T75 flask). Incubate at room temperature for 5 min or until cells have detached from the culture surface. Pipette cells gently to help make a single-cell suspension and inactivate the TrypLE Express by diluting it with 10 mL of RPMI medium (*see Note 17*).

9. Count cells and take a volume of cell suspension that contains 2×10^6 cells, and centrifuge the cell suspension at $200 \times g$ for 10 min.
10. Resuspend the 2×10^6 cells of fibroblasts in 1 mL of RPMI medium and transfer them to one 1.5 mL Eppendorf tube.

**3.3 Isolation
of PBMCs from Patient
Whole Blood
and Preparation
of Cells
for Nucleofection**

1. Collect 10 mL of whole blood in a heparin-coated tube.
2. Prepare the SepMate-50 tube by adding 15 mL of density gradient medium through the central hole in the insert (*see Note 18*).
3. Transfer 10 mL of whole blood to one 50 mL Falcon tube.
4. Dilute the whole blood with an equal volume of blood cell dilution solution and invert the cell sample several times to mix.
5. Add 20 mL of the diluted blood to the pre-prepared SepMate-50 tube by pipetting the solution quickly down the wall of the tube.
6. Centrifuge SepMate-50 tube at $1200 \times g$ for 10 min at room temperature.
7. Harvest the PBMCs by quickly pouring the top layer into a new 50 mL Falcon tube and leave the density gradient medium in the tube (*see Note 19*).
8. Centrifuge the 50-mL Falcon tube containing the top layer at $300 \times g$ for 15 min at room temperature.
9. Remove the supernatant, wash the PBMC pellet with PBS-/-, and centrifuge again at $300 \times g$ for 10 min at room temperature.
10. Add 20 mL of RPMI medium gently to the cell pellet and resuspend the PBMCs. Transfer the cells to a T75 flask and culture overnight in an incubator at 37°C and 5% CO_2 (*see Note 20*).
11. After 24 or 48 h, take 500 μL of cell suspension and count the cells using a dead cell dye such as Trypan Blue to determine the concentration of living cells.
12. Take a volume that contains 2×10^6 cells and centrifuge the cells at $300 \times g$ for 10 min.
13. Resuspend the PBMCs in 1 mL of RPMI medium and transfer them into a 1.5 mL Eppendorf tube.

**3.4 Nucleofection
of Fibroblasts
or PBMCs**

1. For PBMCs, use the P3 Nucleofector kit; and for fibroblasts use the P2 Nucleofector kit. Add the corresponding supplement into the nucleofector solution (4.5:1 ratio of nucleofector solution to supplement). Allow the solution to reach room temperature before use (*see Note 21*).

2. Add 10 mL of RPMI media supplemented with 20 ng/mL of FGF2 to a 10 cm vitronectin-coated dish and allow it to be equilibrated in an incubator at 37 °C and 5% CO₂ for 1 h.
3. When conducting the steps below, pre-warm 5 mL of RPMI medium to 37 °C.
4. Prepare the plasmids by mixing 2.5 µL of each plasmid (from a stock of 1 µg/µL) (pCXLE-hOct3/4-shp53-F, pCXLE-hSK, pCXLE-hUL, and pCXLE-EGFP) to make a total volume of 10 µL (*see Note 5*).
5. Spin down the 1.5 mL Eppendorf tube containing 2 × 10⁶ fibroblasts or PBMCs in a centrifuge at 90 × *g* for 10 min using swing-out buckets to ensure that all cells are pelleted at the bottom of the tube.
6. Remove the media completely, add 100 µL of nucleofection solution to the cells, and resuspend by tapping the tube and gentle pipetting with a p200 tip.
7. Add 10 µL of the plasmid mix to the cell suspension (*see Note 22*).
8. Mix the cells and DNA by pipetting and immediately transfer the cells into the nucleocuvette vessel with a p200 pipette, and then gently tap the nucleocuvette to ensure the cell suspension is at the bottom. Make sure there are no large bubbles in the solution (*see Note 23*).
9. Immediately load the cuvette strip into the 4D-Nucleofector X-Unit and run the program EN-150 for fibroblast cells, or the EO-115 program for PBMCs (*see Note 24*).
10. Incubate the nucleocuvette for 10 min at room temperature.
11. Add 1 mL of pre-warmed RPMI media into the nucleocuvette, using the supplied plastic Pasteur pipette, and gently mix with cells in the cuvette to dilute the cells and to help remove them from the cuvette. Immediately, seed the cells by gently dispersing the cell suspension around the vitronectin-coated 10 cm dish that contains 10 mL of RPMI media supplemented with 20 ng/mL FGF2. Then, grow the cultures inside an incubator at 37 °C and 5% CO₂. After 24 h, examine the cells under a fluorescent microscope to assess transfection efficiency based on GFP expression (*see Note 25*).
12. After 72 h, remove the RPMI media and add 10 mL of the pre-warmed E7 media.
13. Change the E7 media every 2 days for approximately 20 days until iPSC colonies appear, which could take up to 30 days. Once iPSC colonies are visible, at around day 20, replace the E7 medium with the E8 medium (*see Notes 26 and 27*) (Fig. 2).

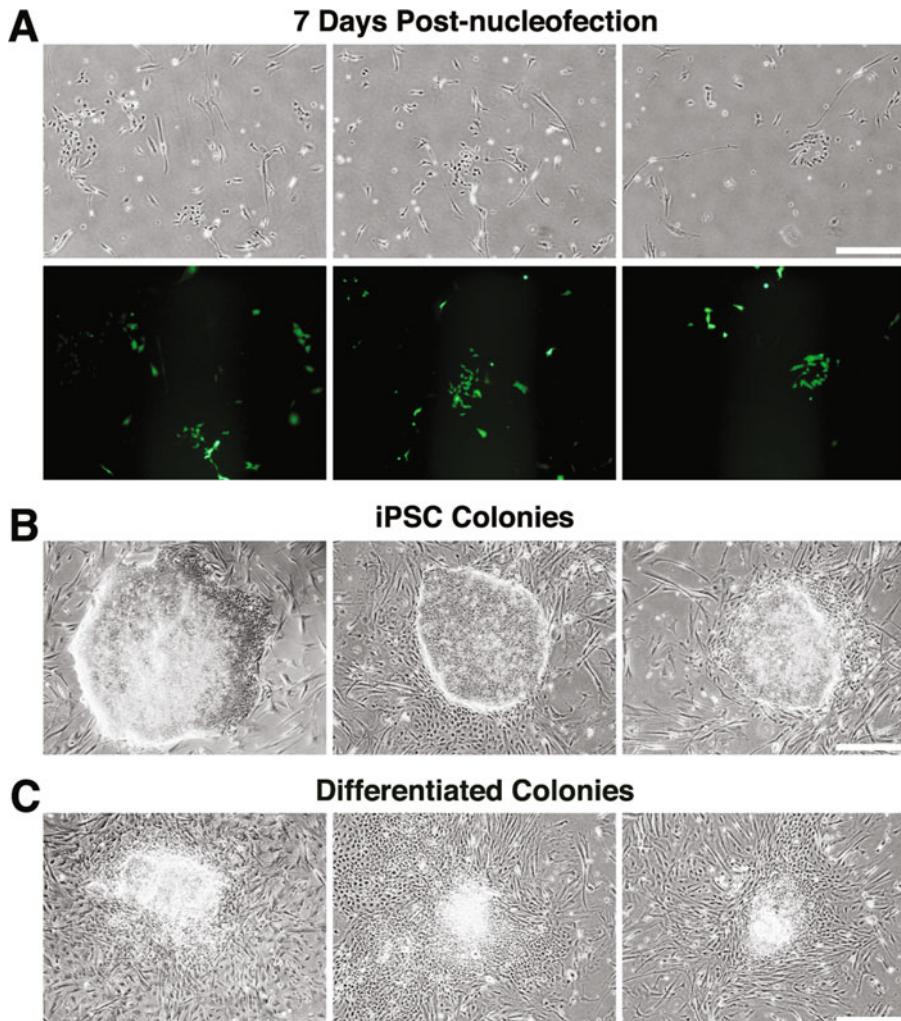


Fig. 2 Reprogramming of human fibroblasts on vitronectin-coated dishes in defined media. **(a)** Examples of cells 7 days post-nucleofection. Small proliferative cells can be seen. GFP fluorescence is shown below bright-field images. **(b)** At day 20–30 colonies with an iPSC morphology can be identified, and some differentiated cells can be seen surrounding the colonies. **(c)** Examples of unsuccessfully reprogrammed colonies that can usually be found in the dish. Scale bar, 500 μ m

3.5 Isolation and Expansion of iPSC Clones on Vitronectin-Coated Dishes

iPSC colonies that have emerged from the reprogramming can be expanded on vitronectin-coated dishes for later freezing, characterization, and long-term culture on vitronectin or human fibroblast feeders (Fig. 2).

- When iPSC colonies are large enough, identify the isolated colonies with good pluripotency morphology, which are 1 mm or more in diameter (Fig. 2b). Cut each colony into 0.5-mm fragments with a 25G needle attached to a 1-mL syringe under a stereomicroscope (*see Note 28*).

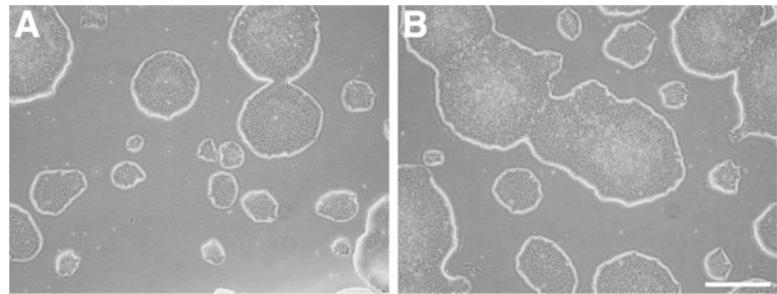


Fig. 3 Expansion of iPSC colonies on vitronectin-coated dishes in the chemically defined media. **(a, b)** iPSC colonies growing on vitronectin-coated dishes in E8 media. Scale bar, 500 μ m

2. Gently lift the fragments off the plastic with the needle tip or gently push the fragments off with a P20 tip attached to a pipette. Transfer all the isolated fragments from one colony into a vitronectin-coated 35 mm dish containing the E8 medium and incubate the cells at 37 °C and 5% CO₂. Do not mix cells from different colonies (*see Notes 29–31*).
3. Change media every day and culture the cells until the colonies grow to a size of approximately 2–3 mm in diameter, which takes around 1 week.
4. Aspirate the spent media, add 1 mL of Gentle Cell Dissociation Reagent to the dish, and then incubate for approximately 5 min (*see Note 32*).
5. Remove the Gentle Cell Dissociation Reagent and add 1 mL of E8 media. Using a P1000 pipette, scrape the colonies off the bottom of the dish. Transfer the cell suspension to a 1.5-mL Eppendorf tube. Using a P1000 pipette, pipette the cell suspension once or twice to break up the colonies to approximately 50–200 μ m in size (*see Note 33*).
6. Plate the cell aggregate mixture on a new vitronectin-coated 35-mm dish containing 2 mL of E8 medium. In 5–7 days, when the colonies are approximately 2 mm in diameter, passage the cells again until an even distribution of colonies are present on the dish, after which the cultures can be passaged every 5–7 days from 1:10 up to 1:50 splits (Fig. 3). After the cells are expanded, the cells are ready for freezing, further characterization, and transferring to feeder dishes for long-term culturing.

3.6 Long-Term Culture of iPSCs by Mechanical Passaging on Fibroblast Feeders

For long-term culture, iPSCs can be maintained on vitronectin-coated dishes in E8 medium (Subheading 3.5) or by manually passaging them weekly on human fibroblast feeders in KSR medium supplemented with FGF2 and Activin A (Fig. 4) (*see Note 34*).

1. The day before passaging, prepare irradiated human fibroblast cells on gelatin-coated organ-culture dish as described in Subheading 3.1.
2. On the day of passaging, remove media from the feeder cells and wash the feeder cells with 1 mL of PBS+/. Remove the PBS+/- and add 1 mL of 37 °C E8 media.
3. Continuing from step 6 of Subheading 3.5, plate the 50–200 µm cell aggregates at a ratio of 1:10 up to 1:50 onto the organ-culture dish with feeders, and incubate the cells at 37 °C and 5% CO₂. Change the E8 media daily.
4. On day 4, remove the E8 media and wash the cells with 1 mL of PBS+/. Add 1 mL of KSR medium supplemented with 15 ng/mL of FGF2 and 10 ng/mL of Activin A and change KSR medium every 2 days (*see Notes 35 and 36*).
5. After a culture of 7 days, under a stereomicroscope scratch the clones manually from the dish by cutting the colony into 0.5 mm fragments with a 25G needle attached to a 1 mL syringe, avoiding the differentiated regions (Fig. 4b). Transfer ten pieces of iPSCs to a fresh feeder layer of mitotically inactivated human fibroblast in KSR medium supplemented with 15 ng/mL of FGF2 and 10 ng/mL of Activin A and place the cell clumps evenly in a circle (Fig. 4).

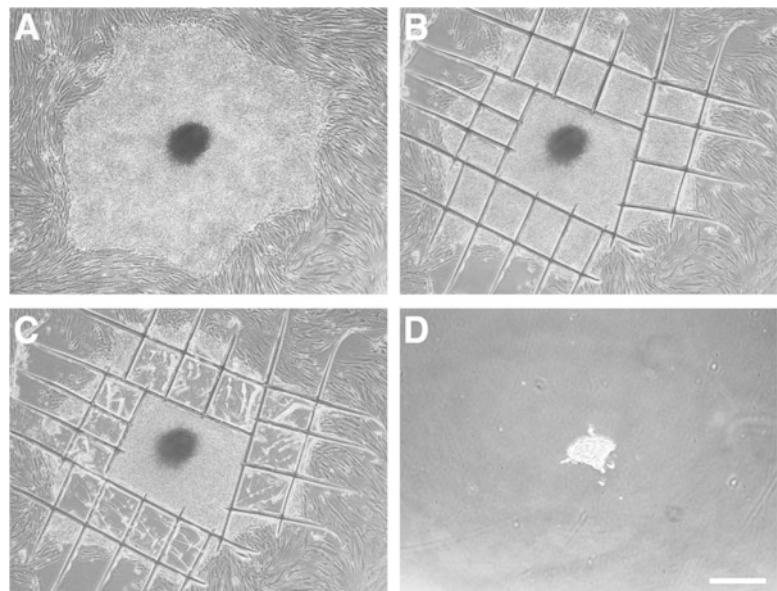


Fig. 4 Manual passaging of iPSC colonies and their expansion on human feeders. **(a)** iPSC colony at day 7 on human fibroblast feeders. **(b)** Undifferentiated regions of the colony have been cut into 0.5 mm fragments. **(c)** Undifferentiated fragments are removed from the dish and transferred to a wash dish. **(d)** A single fragment in a wash dish that was cut and removed from the original iPSC colony. Scale bar, 500 µm

6. Passage the iPSC culture every week to a freshly prepared feeder layer of mitotically inactivated human fibroblasts in KSR medium supplemented with 15 ng/mL of FGF2 and 10 ng/mL of Activin A.
7. When sufficient iPSCs are expanded, assess iPSCs for random integration of transgenes, mycoplasma contamination, pluripotent marker expression, and differentiation potentials, and conduct fingerprint analysis and karyotype analysis (*see Notes 37 and 38*).

4 Notes

1. The organ-culture dishes contain an outer ring that should be filled with PBS–/– or sterile water, which will help maintain the humidity in the culture dish.
2. Purchase FCS that has low endotoxin levels.
3. 2-Mercaptoethanol will only last for 1 month at 4 °C after opening. Cell quality may be affected if 2-mercaptoethanol is used after 1 month from the first use.
4. All plasmids should be at a concentration of 1 µg/µL and dissolved in EB buffer (10 mM Tris-Cl, pH 8.5) or sterile water.
5. EGFP plasmid is optional, and it is only needed if you want to monitor and quantify the transfected cells. Therefore, if you do not add EGFP the total volume of DNA used will be 7.5 µL of plasmids, and it is not necessary to add more of the other plasmids.
6. Growth factors are reconstituted according to manufacturer's instructions. Growth factors can be stored at –80 or –20 °C for 3 months or at 4 °C for 1–2 weeks. Growth factors should be added to media freshly on the day of use.
7. Media can be stored for up to 4 weeks at 4 °C.
8. E7 and E8 medium can be aliquoted into 50 mL tubes and frozen at –20 °C for up to 3 months. Aliquots can be stored for 4 weeks at 4 °C.
9. Dishes can be stored for up to a week at 4 °C. Dishes must not be allowed to dry, and only non-treated dishes should be used with vitronectin for optimal coating.
10. If not used immediately, the unsterilized gelatin stock solution should be frozen immediately at –20 °C for long-term storage. The working gelatin solution can be stored at 4 °C for up to 4 weeks. The dried gelatin-coated dishes should be used within 2 h, and storage for extended time may disrupt the structure of the gelatin.

11. Other fibroblasts can be used; as feeders however, they will need to be tested to determine if they support the iPSCs in an undifferentiated state. iPSCs should be cultured on feeders for at least four passages to test the feeder cell's ability to support pluripotency. Mouse embryonic fibroblasts (MEFs) can be used to support human pluripotent stem cells. They can be prepared from an eviscerated E12.5 to E14.5 fetuses. MEFs will also need to be tested for their suitability to support iPSCs.
12. Fibroblasts can be expanded by sub-culturing and be used as feeders. CCD-1079Sk cells can be expanded up to passage 20 and used as feeders. Expanding the cells beyond passage 20 is possible; however, the fibroblast's ability to support the iPSCs will need to be assessed.
13. The concentration of the cells can vary and will not affect the irradiation time. The limitation will be the size of tube that can fit inside the irradiator. If you do not have access to irradiation equipment, mitotic inactivation can be achieved by incubating the cells, while still attached in the flask, in RPMI medium supplemented with 10 µg/mL of Mitomycin C at 37 °C for 2.5 h.
14. Irradiated feeder cells can be cultured for up to 3 days before iPSC clumps are grown on them. iPSC clumps are cultured for 7 days on the irradiated feeder cells. Therefore, the feeder cells can be readily maintained for approximately 10 days in total. If kept for longer periods of time, the feeder cells will start to die, which will lose the ability to maintain iPSCs.
15. Within a few days, keratinocytes will start to grow out from the skin explants. Fibroblasts can usually be seen after a week. The keratinocytes cannot be maintained in the RPMI media and after a few passages only fibroblasts will be visible.
16. Patient fibroblast cells can also be obtained from biobanks. Low passage human fibroblasts reprogram more efficiently, and during expansion, cells should be passaged before they become confluent. Cells at high density will become quiescence due to contact inhibition.
17. TrypLE Express is used instead of trypsin because we have found that it results in greater survival after nucleofection.
18. When adding the density gradient medium through the central hole in the insert, bubbles may be present in the medium; however, this will not affect the separation.
19. When pouring off the top layer, which contains the PBMCs, do not invert the SepMate-50 tube for more than 2 s because the density gradient medium may come out.
20. The PBMCs grow in suspension and do not attach to the flask.

21. Once the supplement is mixed with the Nucleofector solution, it is stable for only 3 months at 4 °C. Use of old solution after this time is possible; however, the efficiency of the transfection will be reduced. Therefore, only mix together the required volume of the supplement and Nucleofector solution. For one 100 µL cuvette, use 82 µL of Nucleofector solution and 18 µL of the supplement.
22. Plasmids volume should not exceed 10% of the final volume of cell suspension. Therefore, the required concentration of 1 µg/µL is important to ensure that the total volume of plasmids used is kept low.
23. Cells should not be left in the nucleofection solution for an extended time, as this will reduce transfection efficiency.
24. Cell type other than fibroblasts and PBMCs can be reprogrammed; however, the nucleofection program and the nucleofection kit may be different.
25. GFP expression should be approximately 70% or above. If the transfection efficiency is low, the number of iPSC colonies formed will be reduced. If low efficiency is observed, the optimization of the transfection program will be needed.
26. The time that iPSCs first emerge will vary depending on the cell type. After a week, small proliferative cells can normally be seen; however, they do not have a pluripotent morphology. Usually, from day 20 onwards, small iPSC-like colonies will start to appear (Fig. 2).
27. No passaging is required during the reprogramming, which will ensure that each iPSC colony isolated for establishment of iPSC lines will be from unique reprogramming events rather than from multiple sister iPSC colonies arising due to the disaggregation of a single early stage colony.
28. Not all clones need to be picked on the same day, and small clones can be picked up on later days when they become large enough.
29. Use a new tip and needle for each new iPSC colony to be isolated. This is important and will ensure that the cells from the previous colony will not contaminate the new colony.
30. When the iPSC colonies first emerge, it is common to see differentiation around the colony (Fig. 2). The differentiated cells can be left behind during the manual passaging, and it may take several passages to establish an iPSC line without differentiation.
31. Some clones will differentiate, and no iPSC lines can be established from those clones; therefore, approximately 10 iPSC clones should be picked and expanded to ensure the derivation of an iPSC line.

32. The time that the cells are incubated in Gentle Cell Dissociation Reagent may need to be optimized to ensure that cells are broken up to small aggregates but not into single cells.
33. E8 medium can be supplemented with 10 µM of Y27632 (Rho kinase inhibitor), which will help the survival of single cells and small aggregates. If Y27632 is used, for optimal effect, it needs to be added to the iPSCs 2 h before passaging and for 16 h after. The next morning the Y27632 should be removed. The morphology of the iPSCs will change in the presence of Y27632 but will return to a normal pluripotency morphology after it is removed.
34. iPSCs can be maintained long term on vitronectin-coated dishes in E8 media, or on feeder cells in KSR media supplemented with FGF2 and Activin A. E8 media is a chemically defined media and is preferred for clinical uses. Culturing iPSCs on feeders benefits from the extracellular matrix proteins secreted by the feeders, and the media is cost-effective. Furthermore, manual passaging of iPSC on feeders allow for differentiated cells to be easily removed.
35. The E8 medium is only required for the first few days during the transition from vitronectin to human feeders. From this point on KSR medium supplemented with 15 ng/mL of FGF2 and 10 ng/mL of Activin A can be used. If the clones start to differentiate, the E8 media may be required for a longer time.
36. PBS with calcium and magnesium is required when washing iPSC colonies because PBS without calcium and magnesium can cause the iPSCs to detach if left on the culture dish for extended periods of time.
37. Characterization of iPSCs includes assessing for random integration of transgenes, mycoplasma testing, fingerprint analysis, karyotype analysis, expression of pluripotent markers, and the ability to differentiate into the three germ layers. A recent publication by Chen et al. (2019) provides information on the characterization of iPSC clones [13].
38. It is important to assess the clones for random integration of the episomal transgenes. We have found that approximately 10% of the iPSC clones assessed contain episomal plasmid DNA.

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Chapter 10

Generation of Human iPSCs by Protein Reprogramming and Stimulation of TLR3 Signaling

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Abstract

The discovery of induced pluripotent stem cells (iPSCs) allows for establishment of human embryonic stem-like cells from various adult human somatic cells (e.g., fibroblasts), without the need for destruction of human embryos. This provides an unprecedented opportunity where patient-specific iPSCs can be subsequently differentiated to many cell types, e.g., cardiac cells and neurons, so that we can use these iPSC-derived cells to study patient-specific disease mechanisms and conduct drug testing and screening. Critically, these cells have unlimited therapeutic potentials, and there are many ongoing clinical trials to investigate the regenerative potentials of these iPSC-derivatives in humans. However, the traditional iPSC reprogramming methods have problem of insertional mutagenesis because of use of the integrating viral vectors. While a number of advances have been made to mitigate this issue, including the use of chemicals, excisable and non-integrating vectors, and use of the modified mRNA, safety remains a concern. Both integrating and non-integrating methods also suffer from many other limitations including low efficiency, variability, and tumorigenicity. The non-integrating mRNA reprogramming is of high efficiency, but it is sensitive to reagents and need approaches to reduce the immunogenic reaction. An alternative non-integrating and safer way of generating iPSCs is via direct delivery of recombinant cell-penetrating reprogramming proteins into the cells to be reprogrammed, but reprogramming efficiency of the protein-based approach is extremely low compared to the conventional virus-based nuclear reprogramming. Herein, we describe detailed steps for efficient generation of human iPSCs by protein-based reprogramming in combination with stimulation of the Toll-like receptor 3 (TLR3) innate immune pathway.

Key words Recombinant reprogramming proteins, Cell permeable peptides, Human iPSCs, Innate immunity, Toll-like receptors, Nuclear reprogramming

1 Introduction

The discovery that a set of reprogramming factors (OCT4, SOX2, KLF4, and c-MYC) can induce nuclear reprogramming of human somatic cells to induced pluripotent stem cells (iPSCs) is a landmark development in regenerative medicine [1, 2]. Human iPSCs promise an unlimited source of therapeutic cells, without the ethical issues or immune barriers of human embryonic stem cells (ESCs)

[3, 4]. Moreover, when compared to adult stem cells, iPSCs are pluripotent and retain greater proliferation capacity. Human iPSCs can self-renew and can be differentiated into any cell type including cardiomyocytes, endothelial cells, neurons, and hepatocytes [5], thereby making them ideal candidates for disease modeling [6–8] and drug screening [9–12]. Indeed, iPSC-derivatives have recently been used for cell therapy [13]. However, a critical bottleneck in the clinical translation of iPSCs is cell safety because methods that rely on genetic modification of the cells to be reprogrammed may introduce genetic or epigenetic errors during the reprogramming process. To overcome these safety concerns and to increase the yield of safer iPSCs, many laboratories have employed different strategies. These include use of the “non-integrating” vectors [14, 15] or the integrating vectors that can be excised from the genome after reprogramming [16], use of various chromatin modifiers [17], and even a purely chemical approach in the case of mouse iPSC generation [18].

One such approach that can effectively eliminate the risk of modifying the target cell genomes during the reprogramming process is the use of recombinant reprogramming proteins [19, 20]. These reprogramming proteins are made cell penetrating by fusion with a cell permeable peptide (CPP) (or “protein transduction domain,” usually a poly-arginine sequence). CPP is usually linked to the C-terminal of each reprogramming protein, i.e., Oct4, Sox2, Klf4, and c-Myc. Once inside the cells, these CPP-tagged reprogramming factors could readily translocate into the nuclei and reprogram mouse embryonic fibroblasts (MEFs) to “protein-induced pluripotent stem cells” (piPSCs) [19, 20]. These piPSCs are indistinguishable from the classic mouse ESCs. In addition to being a non-integrating method, reprogramming via protein transduction provides a simpler and more controllable approach when compared to other methods of nuclear reprogramming. Despite these advantages, protein-based nuclear reprogramming is inefficient with MEFs as the starting cells [19] when compared to the conventional virus-based integrating approaches. Previous effort to generate human iPSCs using proteins failed. This was perplexing given that these reprogramming proteins are successfully delivered into somatic cells by CPPs, translocate into the nuclei, and elicit transcriptional activation of the downstream targets [21].

In effort to develop effective reprogramming protocols for human cells using CPP-fused proteins, we discovered an intriguing difference in the pattern of gene expression induced by virus-versus protein-based methods of nuclear reprogramming [22]. This observation raised the hypothesis that the virus itself was important in the reprogramming process. Indeed, the additional transduction of the reprogramming cells by an irrelevant retroviral vector led to an efficient protein-based nuclear reprogramming in human fibroblasts, and we found that efficient nuclear reprogramming of

human fibroblasts to pluripotency required activation of the innate immune pathways, including the Toll-like Receptor 3 (TLR3) [22] or RIG-I-like receptors (RLR) [23]. Similarly, modulation of the same innate immune pathways allowed the successful transdifferentiation of human fibroblasts to endothelial cells [24, 25]. Specifically, we discovered that viral vectors themselves, independent of the reprogramming factors that they may encode, stimulated the TLR3 to activate transcriptional pathways, mediated by NF- κ B, IRF3, and IRF7 to cause global changes in epigenetic modifiers [26]. This included downregulation of the histone deacetylase (HDAC) family or upregulation of histone acetyltransferase (HATs) [27], both of which are known to change the chromatin into an open configuration [28, 29], thereby allowing the reprogramming proteins to activate the core pluripotency genes. Subsequently, this discovery allowed us to develop a protocol where human cells can be reprogrammed to iPSCs using protein-based methods, which requires the use of Polyinosinic-polycytidylic acid [poly(I:C)], an FDA-approved TLR3 agonist, along with the Yamanaka factors fused with CPP (OCT4-R11, SOX2-R11, KLF4-R11, and c-MYC-R11).

In this chapter, we provide a detailed protocol for generating human protein-induced pluripotent stem cells (piPSCs) from human fibroblasts by activating the toll-like receptor 3 (TLR3) signaling pathway. We also describe the procedures for iPSC colony isolation and expansion, as well as long-term storage of iPSCs.

2 Materials

2.1 Fibroblast

Growth and Culture

1. Cells: BJ human foreskin fibroblasts.
2. Fibroblast medium: 500 mL DMEM, with 1% GlutaMAX, 10% FBS, 1% penicillin/streptomycin, filter-sterilized.
3. DMEM/F-12 base medium.
4. 6-well cell culture plates.
5. 15- and 50-mL polypropylene conical tubes.
6. Freezing media for fibroblasts: Bambanker (Fisher Scientific, Cat. No. NC9582225).

2.2 Protein-Based iPSC Reprogramming

1. Recombinant reprogramming proteins fused with cell permeable peptides (CPPs): CPP-OCT4, CPP-SOX2, CPP-KLF4, and CPP-cMYC (Applied Biological Materials, Cat. No. 000009P, 000011P, 000001P, 000005P, respectively).
2. Polyinosine-polycytidylic [Poly(I:C)].
3. Essential 8 medium.
4. BD Matrigel.

5. Y27632 (ROCK inhibitor).
6. TrypLE Express.
7. Dimethyl Sulfoxide (DMSO).
8. Dulbecco's phosphate-buffered saline (DPBS, 1×, without Ca^{2+} and Mg^{2+}).
9. Freezing media for iPSCs: Bambanker (Fisher Scientific, Cat. No. NC9582225).
10. Cryovials.

2.3 Equipment

1. Cell culture incubator (37 °C, 5% CO_2 , 85% humidity).
2. Tissue culture hood.
3. Micropipettes.
4. Centrifuge.
5. Inverted cell culture microscope.
6. Autopipettor.
7. Water bath.
8. 6-well cell culture plates.
9. 12-well cell culture plates.
10. 100-mm tissue culture dishes.
11. T75 cell culture flasks.
12. 15- and 50-mL polypropylene conical tubes.
13. Plastic aspiration pipettes (2, 5, 10, 25, and 50 mL).
14. 250- and 500-mL PES media filters.
15. Countess Cell Counter and slides.
16. CoolCell® freezing container.
17. Liquid nitrogen storage.

3 Methods

3.1 Fibroblast Growth and Culture

1. Thaw and plate one cryovial of commercial BJ human fibroblasts in the fibroblast medium in one well of a 6-well plate.
2. Change the medium every 2 days and passage the fibroblasts once they become confluent.
3. To passage the fibroblasts, aspirate medium and wash the cells with 1× DPBS. Add 1 mL TrypLE Express and incubate the cells at 37 °C for 5 min. Following incubation, pipette the TrypLE Express to dissociate the cells and collect them into a 50-mL Falcon tube. Add 4 mL fibroblast media to neutralize trypsin and centrifuge at $300 \times g$ for 5 min. Resuspend the cell pellet in 50 mL of fibroblast medium and plate the cells to a T75 flask. Change medium every 2 days.

4. Once confluent, start to cryopreserve the fibroblasts.
5. Harvest the cells as described above, and resuspend the cells in Bambanker at the concentration of 1×10^6 /mL.
6. Transfer 1 million of fibroblasts in Bambanker into each cryovial (1 mL per cryovial).
7. Place cryovials containing the cells into a CoolCell® freezing container and transfer the CoolCell container inside a -80°C freezer to freeze overnight, and the following day transfer the vials into liquid nitrogen tank for long-term storage.

3.2 Prepare Matrigel-Coated Plates

1. Thaw growth factor-reduced Matrigel overnight at 4°C . Prepare Matrigel aliquots of 250 μL each in 1.5-mL Eppendorf tubes on ice. Store the matrigel aliquots in a -20°C freezer for future use.
2. Thaw one aliquot of Matrigel at 4°C overnight. Add one aliquot (250 μL) of Matrigel to 50 mL of ice-cold DMEM/F12 in a 50-mL Falcon tube. Mix well and add 2 mL of diluted Matrigel solution per well of a 6-well plate.
3. Coat the plates in 37°C cell culture incubator for a minimum of 30 min. These plates can be kept in 37°C incubator overnight for use the next day or stored at 4°C for use up to 1 week.

3.3 Reprogramming of Fibroblasts with Recombinant Reprogramming Proteins and Poly(I:C)

1. Prepare Matrigel-coated dishes 3 days before initiating reprogramming as described above.
2. Thaw a vial of BJ fibroblasts in 37°C water bath and transfer the cells into a 15-mL Falcon tube containing 4 mL of DMEM/F12. Mix well and centrifuge at $300 \times g$ for 5 min. Resuspend the cell pellet in fibroblast medium and plate the cells in a 100-mm Matrigel-coated dish. Culture the cells till confluence ($\sim 2-3$ days) (Fig. 1).

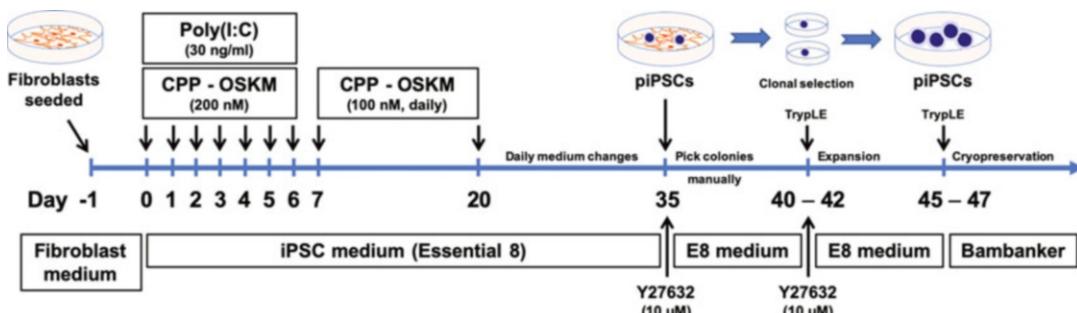


Fig. 1 Schematic of the protocol for protein-induced nuclear reprogramming using transcription factor proteins fused with cell permeable peptides (CPPs) and Poly(I:C). The timeline outlines cell culture conditions and sequential treatments of reprogramming proteins and Poly(I:C) for the different stages of nuclear reprogramming of human fibroblasts to protein iPSCs (piPSCs)

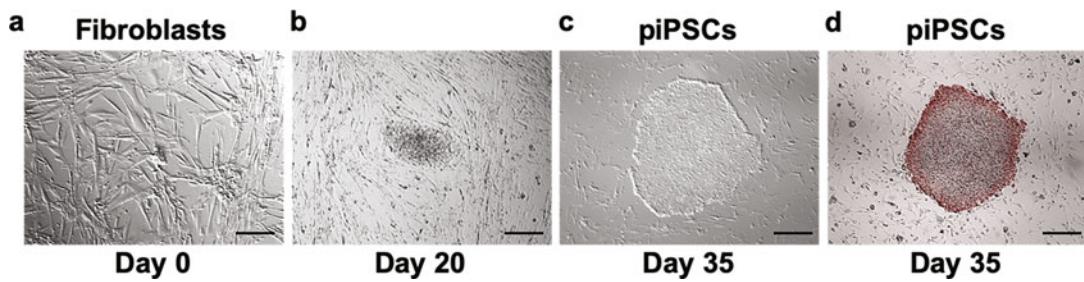


Fig. 2 Protein reprogramming of human fibroblasts to iPSCs. (a–c) Phase-contrast images of three different stages of protein reprogramming. (a) Human fibroblasts at day 0; (b) Early stages of protein-induced nuclear reprogramming at day 20; (c) Appearance of a fully grown human piPSC at day 35. (d) Alkaline phosphatase staining of human piPSCs at day 35

3. Day –1: Passage the BJ fibroblasts with TrypLE as described above. Resuspend the cell pellet in fibroblast medium and count the cells using an automated cell counter. Plate ~100,000–120,000 cells per well of a Matrigel-coated 6-well plate (*see Note 1*).
4. Day 0: Prepare CPP-OSKM cocktail by mixing each protein (CPP-OCT4, CPP-SOX2, CPP-KLF4, and CPP-cMYC) in iPSC medium (E8 medium) to reach a final concentration of 200 nM (*see Note 2*). Add Poly(I:C) to the CPP-OSKM cocktail in E8 medium to reach a final concentration of 30 ng/mL (*see Note 3*). Replace the fibroblast medium in each well of the 6-well plate with 2 mL of the freshly prepared E8 medium containing CPP-OSKM and Poly(I:C).
5. Day 1–6: Change medium each day till day 6 with the freshly prepared E8 medium containing 200 nM CPP-OSKM and 30 ng/mL Poly(I:C) as described above.
6. Day 7–20: Change medium each day with the freshly prepared E8 medium containing 100 nM CPP-OSKM without Poly(I:C).
7. Day 20–25: Change medium each day with E8 medium without reprogramming proteins. Continue to monitor the cells for morphological changes.
8. iPSC colonies should appear at around day 25.
9. Continue medium changes with E8 medium without reprogramming proteins for additional 10 days, and at day 35, piPSC colonies are ready to be counted and picked (Fig. 2).

3.4 Isolation of Colonies and Long-Term Culture of piPSCs

1. At around day 35, the piPSC colonies are large enough to be manually picked under a microscope for propagation. Using a p20 pipette tip, pick six individual piPSC clones and transfer them to six different wells of a Matrigel-coated 12-well plate containing the E8 medium supplemented with 10 µM Y27632 (passage 1) (*see Note 4*).

2. Replace the spent media with the fresh E8 medium without the ROCK inhibitor the next day and continue to culture for around 1 week with daily change of the E8 medium. The individual colonies of piPSCs should start to merge and are ready for the first passage.
3. To passage the P1 iPSCs further, from each well of the P1 iPSC culture, cut one individual colony into many pieces (10–15) with a p20 pipette tip, and transfer all the pieces of the iPSC colony using a p200 pipette into one well of a Matrigel-coated 6-well plate containing the E8 medium supplemented with 10 µM Y27632 (passage 2).
4. Replace the spent media with fresh E8 medium without the ROCK inhibitor the next day and continue to culture for around a week with daily change of the E8 medium. At around day 6, colonies should be confluent and ready for the second passage.
5. Passage the cells at split ratio of 1:12. Aspirate the spent media from iPSCs, and wash briefly with 1 mL PBS (without Ca²⁺ and Mg²⁺). Aspirate the PBS.
6. Add 1 mL of room temperature 0.5 mM EDTA solution to the iPSCs. Incubate for 5 min at room temperature and carefully aspirate the EDTA solution without rinsing the cells.
7. Add 1 mL of E8 medium with 10 µM Y27632 to the well and dislodge the cells by pipetting up and down.
8. Recover the cell suspension in a 50-mL Falcon tube and add 23 mL of E8 medium with 10 µM Y27632.
9. Resuspend gently and add 2 mL of the cell suspension in each well of two Matrigel-coated 6-well plates (passage 3).
10. Passage the piPSC colonies one additional time (passage 4) before cryopreservation (*see Note 5*).

3.5 Cryopreservation of piPSCs

1. After passage 4, piPSCs are ready to be cryopreserved. Follow the protocol to passage iPSCs as mentioned above.
2. Following removal of EDTA solution, resuspend the cells at the concentration of 1 million per mL using the ice-cold Bamban-ker, and aliquot the cells in cryovials at 1×10^6 per vial.
3. Transfer cryovials to a CoolCell container and place it at –80 °C overnight.
4. Transfer the cryovials into liquid nitrogen tank the following day.

4 Notes

1. Plating 80,000–100,000 cells and growing overnight should yield a confluence of 70–75%.
2. CPP-OSKM cocktail should be prepared freshly each day by mixing each reprogramming protein (CPP-OCT4, CPP-SOX2, CPP-KLF4, and CPP-MYC) in the E8 medium to reach a final concentration of 200 or 100 nM.
3. Poly(I:C) can be prepared as a stock solution of 1 mg/mL and aliquoted at the size for one-time daily use from day 0 to day 6. Avoid multiple freeze-thaw of the poly(I:C) aliquots.
4. To ensure that only piPSC clones are picked, the surrounding fibroblasts can be scraped away with a p20 pipette tip.
5. The individual piPSC colonies from each clone of the reprogramming well should not be mixed and should be passaged separately.

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Chapter 11

Generation of Human iPSCs by Reprogramming with the Unmodified Synthetic mRNA

Robert R. Annand

Abstract

Human-induced pluripotent stem cells (iPSCs) are showing great promise for both disease modeling and regenerative medicine. The choice of reprogramming methods have a significant effect on the outcomes of the experiments. Standard methods, such as Sendai viruses, episomes, and the base-modified mRNA have limitations. Here, I describe a method to reprogram human fibroblasts using a cocktail of mRNAs without any base modification that increases reprogramming efficiency, reduces the RNA-associated toxicity, and yields iPSCs ready for expansion and characterization in as short as 10–14 days.

Key words Induced pluripotent stem cells (iPSC), Synthetic mRNA, Cellular reprogramming, Human fibroblasts, Stem cells

1 Introduction

To fulfill the promise of pluripotent stem cells (PSCs) as a source for regenerative medicine and models to understand the basic disease biology and test potential new treatments, a reliable PSC source is required. Induced pluripotent stem cells (iPSCs) have shown the promise to provide this source. The generation of iPSCs from somatic cells involves introducing a set of transgenes, also called reprogramming factors, into the somatic cells to revert the cell fate to a pluripotent state [1, 2]. The commonly used non-integrating vectors to achieve this reversion include Sendai virus vectors [3, 4] and episomal vectors [5]. In addition, both synthetic mRNA [6] and polycistronic, self-replicating RNA viral vectors [7] are commonly used to express reprogramming factors for generation of the footprint-free iPSCs.

All of these reprogramming methods have their limitations. Sendai virus and episomal vectors can be retained by the iPSCs for an extended number of passages, requiring screening to ensure vector clearance, and these methods can lead to the development of genetic abnormalities during reprogramming

[8]. Self-replicating RNA vectors are also retained and require screening for iPSCs without residual RNA vectors. The traditional mRNA methods are laborious, requiring the use of conditioned medium and daily RNA transfections for up to 12 days. In addition, repeated exposure of the reprogramming cells to mRNAs leads to significant, immune-based cytotoxicity, mediated by interferon- α [9, 10], which limits reprogramming efficiency. However, mRNA methods also give high reprogramming efficiency and a lower rate of genetic abnormalities compared to other methods, and mRNA is rapidly cleared from the cells [8], eliminating the need for screening for vector clearance.

To overcome some of the limitations of mRNA-based reprogramming, a new method was developed [11] that delivers high-efficiency reprogramming. Traditionally, mRNAs used for transfection contain the modified bases, such as pseudouridine (ψ) and 5-methylcytosine (5mC) to minimize the toxic responses. In addition, the viral B18 protein is added into the reprogramming media to block the interferon-mediated cellular responses [6]. This new method uses mRNAs without base modification to improve translation efficiency. In addition, mRNAs encoding the B18 protein, as well as two other Vaccinia virus proteins E3 and K3, which also modulate RNA immune response [12], are included in the reprogramming cocktail to minimize the RNA-associated cytotoxicity. Furthermore, a cocktail of microRNAs associated with reprogramming is included to improve reprogramming efficiency. As a result of these improvements, the reprogramming method generates high-quality iPSCs from fibroblasts with only four transfections, without the need for conditioned medium.

In this chapter, I describe a detailed method for generating iPSCs from adult skin fibroblasts using mRNA containing unmodified bases. The protocol starts with fibroblast plating, followed by a series of four transfections with mRNA. After 10–14 days of culture in the NutriStem hPSC XF Medium, colonies develop, and they are picked and replated into individual wells. At that point, the iPSCs are ready for expansion and characterization by methods dictated by the experimental design.

2 Materials

2.1 Caution

These procedures use fibroblasts derived from human tissue. These cells are a potential source of infection with blood-borne pathogens. Prior to beginning the experiment, consult with your institutional biosafety authority for specific guidelines on how to minimize your exposure. Appropriate personal protective equipment (lab coats, gloves, safety glasses, etc.) should be worn throughout these procedures.

2.2 Required Reagents

1. StemRNA™ 3rd Gen Reprogramming Kit (Stemgent REPRO CELL).
2. iMatrix-511 Recombinant Human Laminin-511 E8 Fragments (REPROCELL) (*see Note 1*).
3. NutriStem® hPSC XF Culture Medium (Biological Industries).
4. Lipofectamine® RNAiMAX™ Transfection Reagent (ThermoFisher).
5. Opti-MEM® Reduced Serum Medium (ThermoFisher).
6. Human Serum (*see Note 2*).
7. Advanced-DMEM Medium.
8. Glutamax™ Supplement (ThermoFisher).
9. DPBS, calcium-free, magnesium-free.
10. NutriFreez™ D10 Cryopreservation Medium (Biological Industries).
11. Trypsin-EDTA (0.05%), phenol red.

2.3 Optional Reagents

1. RNAase inactivation reagent, e.g., RNase Zap™ (Ambion).

2.4 Recommend Equipment

1. Multi-gas incubator, equilibrated to 5% O₂: hypoxic incubator (5% O₂) (*see Note 3*).

2.5 Fibroblast Expansion Medium

1. To make a batch of 50 mL, add 5 mL human serum and 0.5 mL Glutamax Supplement to 44.5 mL Advanced DMEM Medium. Store the medium at 4 °C for up to 2 weeks.

3 Methods

This protocol describes the method for reprogramming one well of a 6-well plate. Please scale appropriately if you will use other vessels for reprogramming. A timeline summarizing the reprogramming method is shown in Fig. 1.

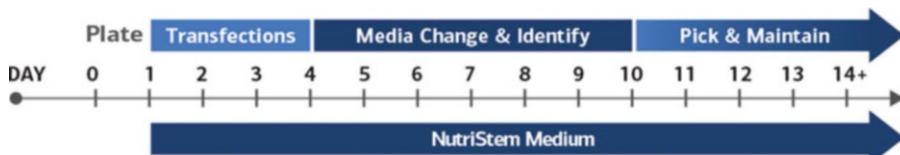


Fig. 1 Fibroblast reprogramming timeline

3.1 NM-RNA Reprogramming Cocktail

The StemRNA 3rd Gen Reprogramming Kit provides sufficient reagents for reprogramming 9 wells in a format of 6-well plates. Use one aliquot of NM-RNA Reprogramming Cocktail as prepared below for each well on each day of transfection.

1. Thaw the three vials of RNA provided in the StemRNA 3rd Gen Reprogramming Kit (OSKMNL NM-RNA; EKB NM-RNA; NM-microRNAs) on ice. Once thawed, keep vials on ice at all times.
2. Briefly centrifuge the vials to collect the contents to the bottom of the vials.
3. Prepare **NM-RNA Reprogramming Cocktail** to reprogram neonatal or adult fibroblasts for a total of four transfections by combining 288 µL OSKMNL NM-RNA, 216 µL EKB NM-RNA, and 50.4 µL NM-microRNAs to a sterile, RNase-free microcentrifuge tube, giving a total volume of 554.4 µL.
4. Divide the mixture into thirty-six 15.4 µL single-use aliquots in sterile, RNase-free microcentrifuge tubes. Store the aliquots at –80 °C for up to 3 months. Avoid additional freeze-thaw cycles. Each aliquot is used for a single transfection of one well in a 6-well plate format.

The daily **NM-RNA Reprogramming Cocktail** aliquots are composed of 0.8 µg OSKMNL NM-RNA, 0.6 µg EKB NM-RNA (total mRNA = 1.4 µg), and 0.4 µg NM-microRNAs per transfection per well (6-well plate format).

3.2 Preparation of Fibroblasts

Prepare a T25 flask of exponentially growing neonatal or adult fibroblasts using standard methods (*see Notes 4 and 5*). Fibroblasts can be thawed and expanded on a T25 flask before seeding for the reprogramming experiments in the **Fibroblast Expansion Medium**.

3.3 Day 0: Plating of Fibroblasts for Reprogramming Experiment

1. Dilute sufficient iMatrix-511 Recombinant Human Laminin-511 E8 Fragments to 2.4 µg/mL in DPBS. Plate 1 mL of 2.4 µg/mL iMatrix-511 substrate (diluted in DPBS) in one well of a 6-well plate and incubate at 37 °C for 1 h before seeding.
2. Remove the culture medium from the T25 flask of exponentially growing fibroblasts to be harvested. Add 5 mL PBS to the flask to wash. Aspirate the PBS.
3. Add 3 mL 0.05% Trypsin/EDTA and incubate for 3–5 min at 37 °C and 5% CO₂.
4. Tap the flask to completely detach the cells from the flask.
5. Add 6 mL **Fibroblast Expansion Medium** to the flask to neutralize the trypsin/EDTA.

6. With a 5-mL pipette, transfer the harvested cell suspension from the flask into a 15-mL conical tube. Pipette up and down gently to disrupt the cell aggregates.
7. Centrifuge the cells for 5 min at $250 \times g$.
8. Remove the supernatant and resuspend the pellet in 1 mL **Fibroblast Expansion Medium**.
9. Count the cells and calculate the live cell density.
10. To the well of the iMatrix-511-coated 6-well plate, add 5.0×10^4 – 7.5×10^4 cells per well in 2 mL of **Fibroblast Expansion Medium** (see Note 6).
11. Incubate the cells overnight in a 37°C , 5% CO_2 , and 21% oxygen incubator.

3.4 Day 1: NM-RNA Reprogramming Cocktail (Overnight Transfection, with Medium Change) (See Note 7)

I recommend that the work surfaces be wiped down with RNase Zap solution to minimize the chance of RNase contamination.

1. Warm up **NutriStem Medium** in a 37°C water bath.
2. Remove the old medium from the well of the fibroblasts seeded with fibroblasts on the iMatrix-511-coated plate from Sub-heading 3.2 (the “reprogramming plate”). Add 2 mL NutriStem Medium to this well.
3. Incubate the fibroblasts in a hypoxic incubator at 37°C , 5% CO_2 , 5% O_2 while preparing the transfection complex.
4. Thaw one single-use aliquot of **NM-RNA Reprogramming Cocktail** at room temperature, then immediately place on ice. Label as tube “A (RNA + Opti-MEM).”
5. Label a sterile, RNase-free 1.5 mL microcentrifuge tube “B (RNAiMAX + Opti-MEM).”
6. To tube A, add 234.6 μL Opti-MEM (tube A already contains 15.4 μL NM-RNA Reprogramming Cocktail).
7. To tube B, add 6 μL RNAiMAX transfection reagent to 244 μL Opti-MEM.
8. Pipette each tube gently three to five times to mix.
9. Using a pipettor, transfer the entire contents of tube B to tube A drop-wise at the meniscus level. Mix by tapping the bottom of the tube. Incubate at room temperature for 15 min.
10. Add 500 μL of the NM-RNA transfection complex solution to the fibroblast-containing well of the reprogramming plate by tilting the plate and pipetting drop-wise into the medium. Mix by moving back-and-forth and side-to-side in the X- and Y-directions, respectively.
11. Return the reprogramming plate to a hypoxic incubator (5% O_2) and incubate overnight.

3.5 Days 2–4:**NM-RNA****Reprogramming****Cocktail (Overnight****Transfection,****with Medium Change)**

On days 2 through 4, replace the spent medium containing the transfection cocktail with fresh NutriStem Medium. Add the transfection complex at the end of the day.

1. **At the beginning of the day**, Warm **NutriStem Medium** in a 37 °C water bath.
2. Remove the old medium from the well in the reprogramming plate. Add 2 mL **NutriStem Medium** into the well.
3. Incubate the cells in a hypoxic incubator at 37 °C, 5% CO₂, 5% O₂ for at least 6 h (*see Note 8*).
4. **At the end of the day**, thaw a single-use aliquot of the **NM-RNA Reprogramming Cocktail** at room temperature, then immediately place it on ice. Label as tube “**A (RNA + Opti-MEM)**.”
5. Label a sterile, RNase-free 1.5 mL microcentrifuge tube as “**B (RNAiMAX + Opti-MEM)**.”
6. To tube A, add 234.6 µL Opti-MEM (tube A already contains 15.4 µL NM-RNA Reprogramming Cocktail).
7. To tube B, add 6 µL RNAiMAX transfection reagent to 244 µL Opti-MEM.
8. Pipette gently three to five times to mix.
9. Using a pipettor, transfer the entire content of tube B to tube A drop-wise at the meniscus level. Mix by tapping the bottom of the tube. Incubate at room temperature for 15 min.
10. Add 500 µL of the NM-RNA transfection complex solution to the well in the reprogramming plate by tilting the plate and pipetting drop-wise into the medium. Mix by rocking in the X- and Y-directions.
11. Return the reprogramming plate to a hypoxic incubator (5% O₂) and incubate overnight (15–18 h).

3.6 Days 5–10 (–14):**NutriStem Medium****Media Changes**

1. Warm **NutriStem Medium** in a 37 °C water bath.

2. Remove the medium from the well in the reprogramming plate and replace with 2 mL fresh **NutriStem Medium**.

3. Return the reprogramming plate to a hypoxic incubator (5% O₂) and incubate overnight.

If the reprogrammed cells show mature iPSC morphology, pickup iPSC colonies as early as day 10 (Fig. 2, *see Note 9*).

3.7 Pick**and Passage iPSCs**

When colonies reach sufficient size and resemble stem cell colonies, they should be picked and replated into individual wells of an iMatrix-511-coated 12-well plate (“Passaging Plate”; *see Note 1*). Picking can be performed with a stereo microscope in either a horizontal flow hood (positive pressure) or a static enclosure.

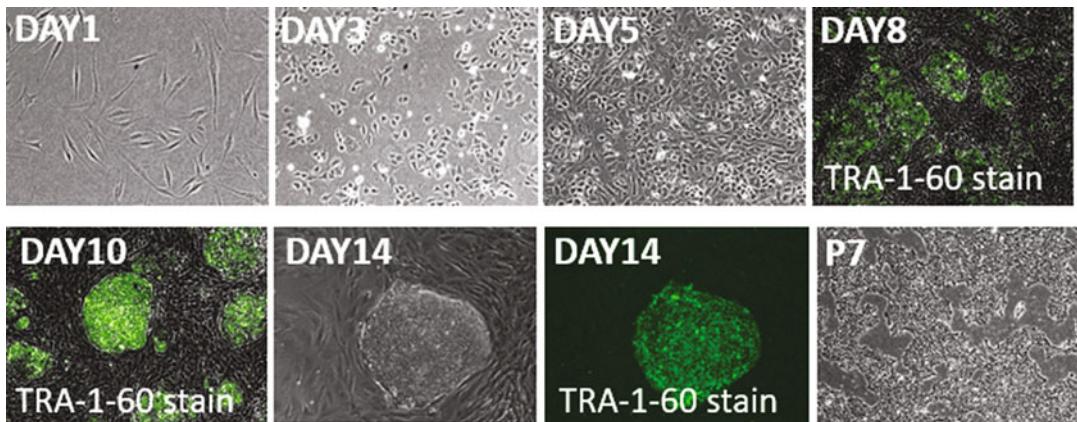


Fig. 2 Primary reprogramming culture morphology progression, resulting from the reprogramming of adult fibroblasts with the StemRNA 3rd Gen Kit on iMatrix-511 in NutriStem hPSC XF Culture Medium. Day 8, 10, and Day 14 primary Fibroblast-RNA-iPSC colonies were identified using Stemgent StainAlive TRA-1-60 antibody and can be isolated from the primary culture between Day 10 and 14. Fibroblast-RNA-iPSCs were expanded on iMatrix-511 in NutriStem hPSC XF Culture Medium

Picking can be done using glass tools made from 9" Pasteur pipettes pulled to a closed, angled end over the controlled flame of an alcohol burner or by using a 10 μ L pipette tip.

All procedures in this picking protocol must be performed in a sterile environment.

1. Coat the appropriate number of wells of a 12-well plate ("Passaging Plate") with 0.5 mL of 2.4 μ g/mL iMatrix-511 in PBS at 37 °C for 1 h prior to picking.
2. Aspirate the medium from 6 wells of the iMatrix-511-coated 12-well Passaging Plate (*see Note 11*).
3. Add 1 mL prewarmed **NutriStem Medium** to each of these 6 wells of the Passaging Plate.
4. Aspirate the medium from each well of the primary 6-well reprogramming plate and replace with 2 mL prewarmed NutriStem Medium.
5. Using a phase-contrast or stereo microscope, locate iPSC colonies based on morphology, and pluripotency marker expression if live staining is conducted before picking.
6. Using a glass picking tool or a 10- μ L pipette tip, gently separate the colony from the surrounding fibroblasts by circling the area to be picked.
7. Using the glass picking tool/pipette tip, gently divide the colony into approximately 3–8 pieces (*see Note 10*). It is important to break the colony into smaller cell aggregates, but not into single cells.

8. Using the glass picking tool/pipette tip, gently and completely detach the colony pieces from the tissue culture plate so that the cell aggregates are freely suspended in the medium.
9. Using a 20- μ L pipettor with a sterile, large-bore tip, transfer the detached colony pieces out of the reprogramming well and into an individual well of the prepared 12-well Passaging Plate. Transfer all of the pieces from one colony into a single well of the Passaging Plate.
10. Repeat the picking and replating process for each iPSC colony. Pick one colony at a time and transfer the cell aggregates from each colony into a different well of the prepared 12-well Passaging Plate. After 6 iPSC colonies have been picked and replated, place both the 12-well passaging plate and the primary reprogramming plate in the hypoxic incubator (5% O₂) for at least 15 min to re-equilibrate.
11. Repeat the process (**steps 1–10**) in increments of 6 iPSC colonies at a time until the desired number of colonies has been picked.
12. Continue to culture the reprogramming (6-well) plate as well as the newly plated picking plate (12-well) until the picked colonies are established.
13. Change **NuTriStem Medium** in both the reprogramming (6-well) and the Passaging Plate (12-well) every day thereafter.

3.8 Maintain and Passage iPSC Cultures

Human iPSC cultures should be monitored and cared for every day, as the overall quality of the culture can change rapidly. Between passages, the cell culture medium must be exchanged every day to provide necessary growth factors for the maintenance of human iPSCs. Human iPSCs are generally passaged every 4–7 days in culture, but the actual passaging schedule and split ratio for each passage will vary depending on the cell culture's quality and growth rate (*see Notes 12 and 13*).

1. Prepare 0.5 mM EDTA solution by adding 10 μ L 0.5 M EDTA to 10 mL of DPBS.
2. To passage, remove the medium from the wells of the 12-well plate of iPSCs. Add 1 mL of 0.5 mM EDTA to each well.
3. Incubate the plate for 3–5 min in a hypoxic incubator. Monitor the cells under a microscope and remove the 0.5 mM EDTA solution once the cells start to detach from the surface.
4. Add 1 mL NuTriStem Medium (prewarmed to 37 °C) by tilting the plate and washing the whole surface in one continuous motion.

5. Plate the cell suspension in an appropriate number of wells of a fresh, iMatrix-511 coated 12-well plate. Swirl to ensure even distribution of cells and return the cells to a hypoxic incubator (*see Notes 13–15*).
6. Change medium every day until next passaging.

3.9 Cryopreservation of iPSCs

iPSCs are cryopreserved by suspension of cell aggregates in NutriFreez D10 Cryopreservation Medium (*see Note 15*). This section describes the cryopreservation of one well of a 6-well plate. Please scale appropriately.

1. Remove the culture medium from the well of the iPSCs to be cryopreserved. Rinse the well with 2 mL DPBS and remove the DPBS.
2. Add 0.5 mL of the 0.5 mM EDTA in DPBS to one well of cells and incubate for 3–5 min in the incubator.
3. Once the cells start to detach, add 1–2 mL of the NutriStem Medium to the well, holding the plate an angle, in one continuous motion to detach the cells. Transfer the cell suspension to a sterile 15 mL centrifuge tube. The contents of multiple wells of the same cells can be combined at this step if desired. Determine a cell count.
4. Centrifuge at $200 \times g$ for 5 min at room temperature. Aseptically decant the supernatant without disturbing the cell pellet.
5. Resuspend the cell pellet in a sufficient volume of ice-cold NutriFreez D10 Cryopreservation Medium to give a cell density of 1×10^6 cells/mL using two to three gentle pipetting. Try to avoid breaking up aggregates more than necessary.
6. Dispense aliquots of cells (typically 1 mL) to prechilled cryovials. Keep cryovials on ice while processing. Gently mix the vials to ensure even distribution.
7. Use a controlled rate freezing system to cool the cells to -80°C at $1\text{--}2^\circ\text{C}/\text{min}$.
8. After overnight freezing at -80°C , transfer the cells to a liquid nitrogen vapor phase storage system for long-term storage.

3.10 Thawing of Cryopreserved iPSCs

1. Briefly warm NutriStem® hPSC XF Medium in a 37°C water bath.
2. Add 9 mL of warmed NutriStem® hPSC XF Medium into a centrifuge tube.
3. Rapidly thaw the cryovial of cells in a 37°C water bath by gently shaking the vial and remove the vial when only a small frozen cell pellet remains. Do not vortex cells.

4. Disinfect the vial by wiping it down with a cloth moistened with 70% ethanol or isopropanol.
5. In a sterile biological safety cabinet, transfer the contents of the cryovial drop by drop into the 9 mL of culture medium in the previously prepared centrifuge tube. Gently shake to continually mix the cells as the new cell droplets are added to the tube.
6. Centrifuge the cells at $200 \times g$ for 5 min. Remove and discard the supernatant.
7. Gently resuspend the cell pellet in NutriStem® hPSC XF Medium or other growth culture media, and plate on a culture vessel coated with iMatrix-511. Incubate at 37 °C.
8. Refresh culture medium 48 h after plating and daily thereafter (see Note 15).

4 Notes

1. Alternatively, Corning® Matrigel® hESC Qualified Matrix, *LDEV-free (Corning) can be used in place of iMatrix-511. Coat the wells according to the manufacturer's instructions.
2. Alternatively, Fetal Bovine Serum (FBS) (Hyclone™ mESC qualified, defined; GE Healthcare) can be used in place of human serum.
3. For plating density with other combinations of serum and matrix, see Table 1.
4. With modifications, such as adjusting the starting medium, the amount of RNA used, and the number of transfections, this method can also be used for reprogramming endothelial progenitor cells derived from blood and epithelial cells derived from urine.

Table 1

Plating conditions for different fibroblast expansion conditions and substrate choices

Recommended plating densities for reprogramming on one well of a 6-well plate (cells per well)		
Fibroblast expansion medium	iMatrix-511	Corning® Matrigel®
A-DMEM + Glutamax 10% human serum	5.0×10^4 – 7.5×10^4	5×10^4 – 1.0×10^5
A-DMEM + Glutamax 10% Hyclone FBS	5.0×10^4 – 7.5×10^4	5.0×10^4 – 7.5×10^4

5. Fibroblasts with lower passage numbers (P2-P6) usually have higher proliferation potential and therefore are easier to transfect with RNA.
6. Depending on the proliferation potential of your cells, 2.5×10^4 – 1.0×10^5 fibroblasts per well can be seeded. See **Note 3** for more information about fibroblast seeding densities using other matrices or serum.
7. I recommend that a hypoxic incubator (5% O₂) be used for reprogramming experiments. Reprogramming in a hypoxic environment results in higher reprogramming efficiency for a variety of reprogramming methods [13]. Alternatively, a standard tissue culture incubator (ambient O₂, 5% CO₂) can be used, but reprogramming efficiency may be lower.
8. Adding recovery time before adding the next dose of transfection complex may decrease the cell toxicity in some cases. At least 6 h is typically sufficient.
9. Cells can be stained with a live-staining pluripotency antibody such as the StainAlive™ TRA-1-60 Antibody (Stemgent REP ROCELL), using the supplier's protocol, to verify pluripotency.
10. Try to pick the inside of the colony without taking the surrounding non-reprogrammed fibroblasts.
11. Work with only 6 wells of the 12-well picking plate at a time to ensure that the 6-well reprogramming plate and the picking plate are not outside of the incubator for an extended time. Return the reprogramming plate (with the established iPSC colonies) and the picking plate (with the freshly plated colonies) to a hypoxic incubator for at least 15 min between sets of 6 wells.
12. Within the first few days of each passage, the proliferating cells grow easily in a monolayer colony. Once the colony becomes large, the proliferating cells begin to pile up, sometimes causing unwanted spontaneous differentiation to occur. It is important to passage the cells before the cultures become overgrown.
13. For the initial passage or two after picking and plating, one well of iPSCs should be split into 2–4 wells on a fresh iMatrix-511-coated plate. For a well-growing culture, one well of iPSCs can be expanded and replated to 8–10 fresh wells during passaging. Not all of these wells may be necessary, as the number of wells and plates can quickly get out of hand.
14. Human iPSCs can be cryopreserved either as clumps/aggregates or as single cells.
15. Try not to disturb the culture plate for the first 24–48 h after passaging or thawing to ensure that the cells attach well to the surface.

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Chapter 12

Reprogramming of Fibroblasts to Human iPSCs by CRISPR Activators

Jere Weltner and Ras Trokovic

Abstract

CRISPR-mediated gene activation (CRISPRa) can be used to target endogenous genes for activation. By targeting pluripotency-associated reprogramming factors, human fibroblasts can be reprogrammed into induced pluripotent stem cells (iPSCs). Here, we describe a method for the derivation of iPSCs from human fibroblasts using episomal plasmids encoding CRISPRa components. This chapter also provides procedure to assemble guide RNA cassettes and generation of multiplexed guide plasmids for readers who want to design their own guide RNAs.

Key words CRISPR, Cas9, CRISPRa, Reprogramming, Pluripotency, iPSC

1 Introduction

Somatic cells can be reprogrammed to pluripotency by ectopic expression of a set of reprogramming factors [1]. Reprogramming factors are conventionally delivered into the reprogramming cells as transgenes. Other means that do not rely on transgenic reprogramming factors have also been reported. These include, for example, reprogramming by RNA interference and small molecular inhibitors [2–4]. CRISPR-mediated gene activation (CRISPRa) relies on sequence-specific targeting of a gene promoter for its activation by a deactivated Cas9 ribonucleoprotein (dCas9), fused with an activator domain [5–8]. Using this approach, any individual silenced endogenous gene can be activated specifically. CRISPRa may therefore be useful in pluripotent reprogramming by its activation of pluripotency-associated factors, which are silenced in the starting cell population. Pluripotent reprogramming with the CRISPRa system has been reported recently for both mouse and human [9, 10]. CRISPRa has the capacity to specifically activate the master pluripotency genes and subsequently the pluripotency regulatory networks.

The most commonly used factors for human pluripotent reprogramming are OCT4 (POU5F1), SOX2, KLF4, MYC, NANOG, and/or LIN28A [11–13]. These factors can be delivered as non-integrating vectors to generate human iPSCs with their genomes intact. Commonly used non-integrating approaches for reprogramming include Sendai Viral replicons, replicating episomal plasmids, and modified mRNA transfections [14–16]. The method described herein utilizes the replicating but transient episomal plasmids to deliver a dCas9 activator and multiplexed guide RNA cassettes [10, 17]. Gene activation is achieved by using a dCas9 fused with a multimeric VP16 acidic activation domain (dCas9VP192) [18], and a plasmid with the U6 promoter to drive expression of guide RNAs targeting the promoters of *OCT4*, *SOX2*, *KLF4*, *MYC*, and *LIN28A*. An additional plasmid containing guide RNA sequences targeting a conserved EGA-enriched Alu-motif (EEA-motif) is included to enhance the reprogramming efficiency [10, 19]. The core protocol for reprogramming human fibroblasts with CRISPRa is described in the first part of the methods section (Subheadings 3.1–3.7). Additionally, we have included methods for assembling and cloning of multiplexed guide RNA plasmids for readers to prepare their own targeting constructs (Subheadings 3.8 and 3.9). The second part has been included in the case that the core protocol needs to be modified, for example, to generate new guides targeting additional genes.

2 Materials

2.1 Cells and Episomal Vectors for Reprogramming

1. Human foreskin fibroblasts (HFF).
2. Activator plasmid: pCXLE-dCas9VP192-T2A-EGFP-shP53, Addgene plasmid #, 69535 (*see Note 1*).
3. Guide RNA plasmid targeting the pluripotency genes: GG-EBNA-OSK2M2L1-PP, Addgene plasmid #, 102902.
4. Guide RNA plasmid targeting the EEA-motif: GG-EBNA-EEA-5guides-PGK-Puro, Addgene plasmid #, 102898.

2.2 Media and Reagents

1. Essential 8 (E8) Medium for human iPSC culture.
2. Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium.
3. TrypLE Select cell dissociation reagent.
4. Basement membrane matrix (Matrigel), growth factor reduced, phenol red free.
5. EDTA iPSC dissociation solution: 0.5 mM ethylenediaminetetraacetic acid (EDTA) in calcium/magnesium-free PBS.

6. Fibroblast growth medium: Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 2 mM GlutaMAX, and 100 units/mL penicillin-100 µg/mL streptomycin.
7. Human embryonic stem cell medium (hESC-medium): KnockOut DMEM (KO-DMEM), 20% KnockOut Serum replacement (KOSR), 1% GlutaMAX, 0.1 mM beta-mercaptoethanol, 1% MEM Non-essential Amino Acids (NEAA), 6 ng/mL basic fibroblast growth factor (bFGF).
8. Induction medium: 1:1 mixture of the fibroblast growth medium and hESC-medium, supplemented with 0.25 mM sodium butyrate (NaB).

2.3 Equipment

1. Electroporation instrument: Neon transfection system, Neon transfection system 100 µL Tip Kit including Buffer R, Buffer E2, and Neon transfection tubes (*see Note 2*).
2. Cell culture biosafety cabinet.
3. Inverted microscope.
4. Fluorescent microscope.
5. Cell culture vessels: 10-cm dishes, 24-well plates, and T-150 flasks.
6. Countess Automated Cell Counter: counting chamber slides, trypan blue dye (*see Note 2*).
7. Centrifuge.
8. Tissue culture incubator.
9. Serological pipettes.
10. Centrifuge tubes (15 and 50-mL).
11. Hybridization Oven.

2.4 Plasmid Preparation

1. Luria-Bertani (LB) broth with 50 mg/mL ampicillin.
2. Midiprep plasmid purification kit.

2.5 Characterization Reagents (Immuno-cytchemistry)

1. PFA solution: 4% paraformaldehyde (PFA) in PBS.
2. Permeabilization solution: PBS with 0.5% Triton X-100.
3. Blocking solution: Ultravision Protein Block.
4. Staining buffer: PBS with 0.1% Tween-20.
5. Antibodies for pluripotency markers: OCT4, SOX2, LIN28A, and TRA-1-60 (Tables 1 and 2).

2.6 Reagents Used for PCR Detection of Episomal Vectors

1. Direct PCR Lysis Reagent (*see Note 2*).
2. Proteinase K, 20 mg/mL stock.
3. Phusion DNA Polymerase.
4. PCR buffer 5×: Phusion HF Buffer 5×.

Table 1
Primary antibodies

Antibody	Species	Dilution	Supplier
OCT4	Rabbit	1:500	Santa Cruz Biotechnology
SOX2	Rabbit	1:500	Cell Signaling
LIN28	Rabbit	1:500	Cell Signaling
TRA-1-60	Mouse	1:200	Thermo Scientific

Table 2
Secondary antibodies

Antibody	Species	Dilution	Supplier
Anti-Mouse IgG (H+L) Alexa Fluor 488	Donkey	1:500	Invitrogen
Anti-Mouse IgG (H+L) Alexa Fluor 594	Donkey	1:500	Invitrogen
Anti-Rabbit IgG (H+L) Alexa Fluor 488	Donkey	1:500	Invitrogen
Anti-Rabbit IgG (H+L) Alexa Fluor 594	Donkey	1:500	Invitrogen

5. dNTP mix, 2.5 mM.
6. Dimethyl sulfoxide (DMSO).
7. Betaine, 5 M.
8. Forward and reverse primers for PCR (Table 3).

2.7 Multiplexed Guide Assembly

1. U6 template plasmid: pX335-U6-Chimeric_BB-CBh-hSpCas9n(D10A), Addgene plasmid #, 42335.
2. Polymerase: Phusion High-Fidelity DNA polymerase.
3. PCR buffer 5×: Phusion HF Buffer 5×.
4. dNTP mix, 2.5 mM.
5. Golden Gate Restriction enzyme: Esp3I or BsmBI.
6. T4 DNA ligase.
7. T4 ligase buffer.
8. Dithiothreitol (DTT), 100 mM.
9. Golden Gate destination plasmid: GG-dest, Addgene plasmid #, 69538.
10. DNA agarose gel extraction kit, and PCR purification kit.
11. Chemically competent *E. coli*, DH5α.
12. LB agar plates with 100 mg/mL ampicillin.
13. Oligonucleotide sequences used in guide assembly PCR reactions are presented in Table 3.

Table 3
Oligo sequences

Name	Sequence 5' to 3'	Use
5pTailedU6promFw	GTA AAAACGACGCCAGTGagggcctattccatgttc	Tailed U6 promoter PCR
U6promRv	GGT GTTT CGTCCTTCCAC	Tailed U6 promoter PCR
TermRv80bp	AAAAAAAAGccccggactcggtggacttttcaagtgtataacggactagcctttaacttgcataTTTCragtcctaaac	Tailed tracR template PCR
3pTailedTerm80bpRv	AGGAAAACAGCTATGACCATTGAAAGccccggactcggtggccac	Tailed tracR template PCR
Term80 Fw	gttttagagtaGAAAtageaag	Tailed tracR template PCR
1_aggc_Fw	ACTGAATTGGATCCTCGAGCGTCTC ACCCTG TAAACGACGGCCAGT	Golden Gate PCR assembly position 1
1_aggc_Rv	CATGGGGCGCGTCGACAGATCTCGTCTC ACATGA GGAAACAGCTATGAC CATG	Golden Gate PCR assembly position 1
2_aggc_Fw	ACTGAATTGGATCCTCGAGCGTCTC ACATGG TAAACGACGGCCAGT	Golden Gate PCR assembly position 2
2_aggc_Rv	CATGGGGCGCGTCGACAGATCTCGTCTC AGTCCA GGAAACAGCTATGAC CATG	Golden Gate PCR assembly position 2
3_aggc_Fw	ACTGAATTGGATCCTCGAGCGTCTC AGGACG TAAACGACGGCCAGT	Golden Gate PCR assembly position 3
3_aggc_Rv	CATGGGGCGCGTCGACAGATCTCGTCTC ACTGGA GGAAACAGCTATGAC CATG	Golden Gate PCR assembly position 3
4_aggc_Fw	ACTGAATTGGATCCTCGAGCGTCTC ACCAGG TAAACGACGGCCAGT	Golden Gate PCR assembly position 4
4_aggc_Rv	CATGGGGCGCGTCGACAGATCTCGTCTC AAACAA GGAAACAGCTATGAC CATG	Golden Gate PCR assembly position 4
5_aggc_Fw	ACTGAATTGGATCCTCGAGCGTCTC ATGRTG TAAACGACGGCCAGT	Golden Gate PCR assembly position 5

(continued)

Table 3
(continued)

Name	Sequence 5' to 3'	Use
5_aggc_Rv	CATGGGGCCGGTCGACAGATCTCGTCTCACGTAAACAGCTATGAC CATG	Golden Gate PCR assembly position 5
Generic guide oligo	GTTGAAAGGACGAAACACCCG NNNNNNNNNNNNNNNNNN GTTTAGAGC TAGAAAATAG	Generic guide oligo sequence
OCT4 guide 1	GTGGAAAGGACGAAACACCCG GGGGGAGAAACTGAGGGCGA GTTTAGAGCTA GAAATAG	Golden Gate PCR assembly
OCT4 guide 2	GTGGAAAGGACGAAACACCCG GTGGTGCATGGTCTG GTTTAGAGCTA GAAATAG	Golden Gate PCR assembly
OCT4 guide 3	GTGGAAAGGACGAAACACCCG GACACAACCTGGGCCCTCC GTTTAGAGCTA GAAATAG	Golden Gate PCR assembly
OCT4 guide 4	GTGGAAAGGACGAAACACCCG GGCACACAGTGGCCAGAGGTCTG GTTTAGAGCTA GAAATAG	Golden Gate PCR assembly
OCT4 guide 5	GTGGAAAGGACGAAACACCCG TCTGTGGGGACCTGCACTG GTTTAGAGCTA GAAATAG	Golden Gate PCR assembly
T7_Fw	TAATACGACTCACTATAAGGG	GG-dest insertion sequencing
SP6_Fw	ATTAGGTGACACTATAG	GG-dest insertion sequencing
pEP4-SF1-oriP	TTCCACGAGGGTAGTGAAACC	oriP episome detection
pEP4-SR1-oriP	TCGGGGTGTAGAGACAAC	oriP episome detection
pEP4-SF2-oriP	ATCGTCAAAGCTGCACACAG	EBNA1 episome detection
pEP4-SR2-oriP	CCCAGGAGTCCAGTAGTCA	EBNA1 episome detection
dCas9-F	AAACAGCAGATTTCGCCCTGGA	dCas9 episome detection
dCas9-R	CTGCTCTGCACCTCGGTCTT	dCas9 episome detection

3 Methods

The plasmids used in the core reprogramming protocol are all available on Addgene (http://www.addgene.org/Timo_Otonkoski/). The core reprogramming protocol, Subheadings 3.1–3.7, can be used as described in the text to reprogram human skin fibroblasts. If the core protocol needs to be modified to better address specific research questions, the cloning of additional guide plasmids suitable for human fibroblast reprogramming experiments is explained later in Subheadings 3.8 and 3.9.

3.1 Preparation of Plasmids for Reprogramming

1. Grow overnight cultures of *E. coli* with the activator plasmid, pluripotency factor guide plasmid and EEA-motif guide plasmid in LB broth with ampicillin at 37 °C in a shaker.
2. Purify the plasmids using the protocol for the plasmid purification kit of your choice.
3. Measure the plasmid DNA concentrations and adjust the final concentration to 600 ng/µL or above.
4. Prepare plasmid mixtures for electroporation with a total amount of 6 µg of plasmid DNA per electroporation. Mix 2 µg of the activator plasmid, 2 µg of the pluripotency factor guide plasmid, and 2 µg of the EEA-motif guide plasmid for each 1 million cells to be electroporated. The volume of the plasmid mixture should not exceed 10 µL in order to not dilute the electroporation buffer (Buffer R).

3.2 Preparation of Matrigel-Coated Cell Culture Vessels

Matrigel-coated cell culture plates are needed to support formation of iPSC colonies and culture of the iPSC clones for their expansion. Matrigel-coated vessels can be prepared beforehand and stored in 4 °C for up to a month if sealed well with parafilm to prevent the Matrigel from dehydrating.

1. Prepare Matrigel aliquots by thawing Matrigel slowly overnight on ice at 4 °C to prevent gelling. Divide Matrigel into 200-µL aliquots and store them in a –20 °C freezer.
2. Thaw an aliquot of Matrigel on ice at 4 °C.
3. Dilute one aliquot of Matrigel (200 µL) into 40 mL of the ice-cold serum-free DMEM medium (final dilution of 1:200).
4. Coat cell culture dishes/plates with the diluted Matrigel solution. Coat a 10-cm cell culture dish with 8 mL and 24-well plates with 500 µL of Matrigel solution per well.
5. Coat the vessels at room temperature for 2 h. After incubation, plates/dishes can be wrapped with parafilm, and stored in 4 °C, or used immediately for cell plating.

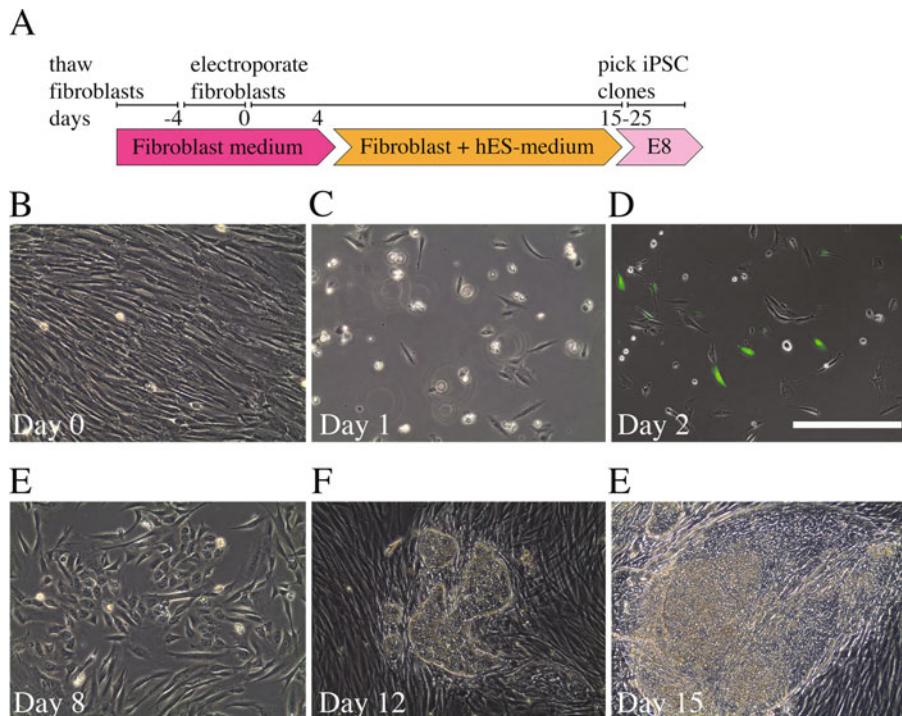


Fig. 1 CRISPRa reprogramming. **(a)** Schematic representation of the CRISPRa reprogramming timeline. **(b)** Confluent HFF culture at day 0 of reprogramming before electroporation. **(c)** HFFs 1 day after electroporation. **(d)** GFP expression in HFFs 2 days after electroporation. GFP is included in the activator plasmid. Scale bar 400 μ m. **(e)** Initial colony formation at day 8 of reprogramming. **(f)** Formation of tightly packed small colonies at day 12 of reprogramming. **(g)** CRISPRa iPSC colony that is big enough for picking at day 15 of reprogramming

6. Immediately before use, remove Matrigel solution and replace it with cell culture medium.

3.3 Preparation of Fibroblasts for Reprogramming

The schematic outline of the episomal plasmid CRISPRa reprogramming protocol is presented in Fig. 1a (see Note 3).

Day -10

1. Quickly thaw a vial of HFF cells and transfer the cells into a 15-mL tube containing 10 mL of fibroblast growth medium. Centrifuge the cells for 4 min at 200 \times g to remove the freezing medium with DMSO.
2. Aspirate the supernatant and resuspend the cell pellet into 10 mL of fibroblast growth medium. Plate HFFs onto a 10-cm culture dish. Allow the cells to recover and grow at 37 °C, 5% CO₂ until the cells reach confluence.

Day -4

3. Passage fibroblasts into T-150 cell culture flasks at 500,000 cells per flask in 25 mL of fibroblast growth medium.

4. Allow cells to grow in T-150 flasks at 37 °C, 5% CO₂ until they are just reaching confluence (Fig. 1b) (*see Note 4*). It should take approximately 4 days for the cells to reach confluence.

Day -1

5. Prepare the plasmid mixtures and Matrigel-coated dishes as described in Subheadings 3.1 and 3.2.

Day 0

6. Remove the Matrigel solution from the dishes and add 10 mL of fibroblast growth medium per 10-cm cell culture dish. Put the Matrigel-coated dishes into a 37 °C incubator to pre-warm the medium.

3.4 Reprogramming of Human Fibroblasts with the Episomal CRISPRa Plasmids

Keep the cells in a 37 °C incubator with 5% CO₂ all the time unless stated otherwise.

Day 0

1. Aspirate the cell culture medium from the T-150 flasks.
2. Rinse the cells once with PBS.
3. Remove PBS and add 6 mL of TrypLE Select per T-150 flask to dissociate the cells. Incubate cells at 37 °C for 3 min. Tap the flasks sharply to detach the rest of the cells.
4. Add fibroblast growth medium into the T-150 flasks to inactivate the dissociation reagent. Collect the cells in a 50-mL conical centrifuge tube and pellet the cells by centrifugation at 200 × g for 4 min.
5. Remove the supernatant, and resuspend the cell pellet in 3–10 mL of PBS (*see Note 5*).
6. To count the cells, take 10 µL of the fibroblast suspension prepared above, and mix with 10 µL of trypan blue in a 1.5-mL tube. Take 10 µL of the cells in trypan blue solution and put them into one chamber of the Countess cell counter slide, and count the cells. Calculate the cell density for cell samples prepared at **step 5** above.
7. Based on cell counting in **step 6** above, take 1 million of cells from the tube in **step 5** for one electroporation of the fibroblasts and centrifuge the cells at 200 × g for 4 min.
8. Prepare the Neon transfection tube by adding 3 mL of electroporation Buffer E2 into the Neon transfection tube and insert the Neon transfection tube into the Neon pipetting station.
9. Remove PBS carefully from the pelleted fibroblast cells from **step 7** above (*see Note 6*).
10. Resuspend the fibroblasts into Neon resuspension Buffer R. Add 100 µL of Buffer R per 1 million of fibroblasts and transfer the resuspended fibroblasts into a 1.5-mL tube.

11. Add 6 µg of plasmid DNA for 1 million of cells to be electroporated to the fibroblast cell suspension from **step 10**. Mix the DNA with the cells carefully (*see Note 7*).
12. Pipette 100 µL of the cell and plasmid suspension in Buffer R from **step 11** into a 100-µL Neon electroporation tip. Insert the tip into the Neon pipetting station containing the Neon transfection tube with Buffer E2 from **step 8**. Electroporate the cells using the setting of 1650 V, 10 ms, and 3× pulse. Plate the electroporated cells (1 million) directly onto a pre-warmed, Matrigel-coated 10-cm cell culture dish with 10 mL of fibroblast growth medium (*see Note 8*).
13. To test activation efficiency of the target genes with immunocytochemical staining, instead of transferring the cells onto a 10-cm dish as in **step 12**, pipette the electroporated cells into a 15-mL tube with 13 mL of fibroblast growth medium. Seed 500 µL of cell suspension per well onto a 24-well plate. The staining method is described in Subheading **3.6**.

Day 1

14. Change fibroblast growth medium for the electroporated cells to remove cells that have died of the electroporation. An example of cells 1 day post electroporation is presented in Fig. **1c**.

Day 2

15. Check the expression of GFP in the electroporated cells with a fluorescent microscope. An example of GFP positive cells at day 2 of induction is presented in Fig. **1d**.

Day 4

16. Replace the fibroblast growth medium with the induction medium on day 4 of reprogramming.
17. Change induction medium every other day until iPSC clones are ready for picking.
18. If target gene activation is to be verified by immunocytochemical staining, the 24-well plate with the reprogramming fibroblasts can be fixed on day 4 as described in Subheading **3.6**.
19. Monitor the reprogramming process daily by observing morphological changes of the cells and colony development (Fig. **1e, f**) (*see Notes 9 and 10*).

Day 14

20. Prepare Matrigel-coated 24-well plates as described in Subheading **3.2**.

Day 15 Onwards

21. Replace Matrigel solution in the Matrigel-coated 24-well plates with 0.5 mL of E8 medium per well.

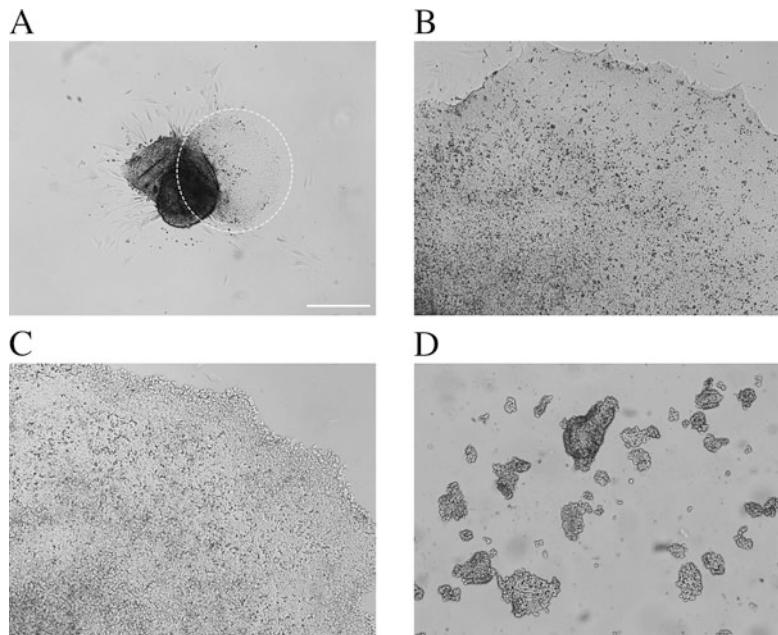


Fig. 2 Passaging CRISPRa iPSC. **(a)** CRISPRa iPSC colony 1 day after picking. The dashed line marks the outgrowth of the cells from the initial attached colony. Scale bar 400 μ m. **(b)** First passage iPSC colony that is big enough for further passaging. **(c)** CRISPRa iPSC colony treated with EDTA solution. Notice small holes forming between cells within the colony. **(d)** CRISPRa iPSC colony after dissociation with pipette tip and gentle trituration demonstrating good-sized cell clusters for passaging

22. Starting from day 15, iPSC colonies should be large enough for picking (Fig. 1g).
23. To establish iPSC clones, cut around an iPSC colony with a scalpel under a dissection microscope and lift the iPSC colony gently off the cell culture dishes with the scalpel blade. Pick the detached colony with a pipette and plate the iPSC clumps in 0.5 mL of E8 media into one Matrigel-coated well of a pre-warmed 24-well plate for the first passage of iPSC clones (*see Note 11*).
24. Incubate the colonies at 37 °C, 5% CO₂ overnight to allow the iPSC clusters to attach.
25. The next day, check that the iPSC clusters have attached to the plate using a microscope, and then replace the spent media with 0.5 mL of fresh E8 media (*see Note 12*). An attached iPSC colony 1 day after picking is shown in Fig. 2a.
26. Change the E8 medium (0.5 mL/well) every other day (*see Note 13*).

3.5 Passaging CRISPRa iPSC Clones for Expansion and Characterization

When the cloned iPSC colonies are large enough (Fig. 2b), passage the cells onto Matrigel-coated 24-well plates for immunocytochemical staining, and onto Matrigel-coated 6-well plates for expansion.

1. To passage the cells, remove the cell culture medium.
2. Rinse cells once with PBS (0.5 mL/well).
3. Add 250 µL of EDTA solution to the cells per one well of a 24-well plate.
4. Incubate the cells with the EDTA solution for 3–5 min at 37 °C or until small holes start to form in the middle of the colonies and the colony edges start to detach (Fig. 2c) (see Note 14).
5. Carefully remove the EDTA solution from the cells and add E8 medium (0.5 mL/well).
6. Scrape the colonies off the plates with a 1-mL pipette tip. Avoid pipetting the cells too much to prevent the cell clumps from breaking into pieces too small. Good-sized cell clumps are shown in Fig. 2d (see Note 15).
7. Plate the cells onto fresh Matrigel-coated plates in E8 medium (2 mL/well).
8. Allow the cells to attach overnight.
9. Change E8 medium every other day.

3.6 Immuno-cytochemical Staining of CRISPRa Cells

Prepare the cells for staining as described in steps 1–18 of Subheading 3.4 for the reprogramming fibroblasts 4 days post electroporation of CRISPRa plasmids, or as described in Subheading 3.5 for established iPSC clones.

1. Rinse cells three times with PBS (0.5 mL/well) (see Note 16).
2. Remove the residual liquid using a 1-mL micropipette.
3. Fix the cells by adding 250 µL of 4% paraformaldehyde in PBS for 15 min at room temperature (see Note 17).
4. Aspirate fixative and wash three times with 250 µL of PBS for 5 min each time.
5. Aspirate PBS and permeabilize cells with 250 µL of permeabilization solution for 10 min (room temperature) (see Note 18).
6. Remove permeabilization solution and wash two times with 250 µL of PBS for 5 min each time.
7. Remove PBS and block epitopes with two drops (see Note 19) of UV blocker for 10 min.
8. Aspirate UV blocker and incubate samples with primary antibody diluted in 150 µL of staining buffer. Incubate plates overnight at 4 °C.

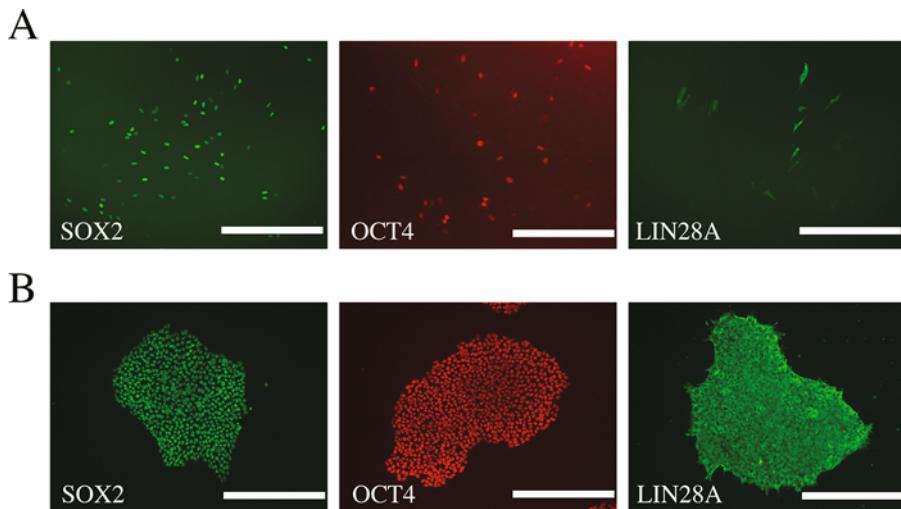


Fig. 3 Example stains of CRISPRa target genes. **(a)** Expression of SOX2, OCT4, and LIN28A in CRISPRa electroporated HFFs at day 4 of reprogramming. Scale bar 400 μ M. **(b)** Expression of SOX2, OCT4, and LIN28A in CRISPRa iPSC clones at passage 2. Scale bar 400 μ M

9. Aspirate primary antibody and wash three times with 250 μ L PBS for 5 min each time (*see Note 20*).
10. Incubate cells with fluorochrome-conjugated donkey anti-mouse IgG antibody diluted in 150 μ L of staining buffer (1:500 antibody dilution) for 30 min in dark at room temperature (*see Note 21*).
11. Aspirate the secondary antibody and wash three times with 250 mL of PBS for 5 min each time.
12. Aspirate all liquid and add two drops (approximately 100 μ L) of DAPI solution (*see Note 22*).
13. Examine and take images using fluorescence microscope.

Examples of activated gene expression at day 4 and passage 2 iPSC clones stained for OCT4, SOX2, and LIN28A are presented in Fig. 3.

3.7 PCR Detection of Reprogramming Vectors

To detect the presence of reprogramming vectors, CRISPRa iPSC colonies are assayed for oriP, EBNA-1, and dCas9 sequences using PCR. PCR primers are listed in Table 3 (*see Note 23*).

1. Aspirate medium from one well of a 24-well plate of CRISPRa iPSCs. Lyse the cells directly in the well in 100 μ L of Direct PCR Lysis Reagent. Collect the cell lysate into a 1.5-mL Eppendorf tube.
2. Add 5 μ L of 20 mg/mL Proteinase K solution to the lysed cells.

Table 4
Reprogramming vector detection PCR

Reagent	Vol (μL)
5 × Phusion HF green buffer	4
2.5 mM dNTPs	1.6
100% DMSO	0.2
10 μM forward primer	1
10 μM reverse primer	1
2 U/μL Phusion DNA Polymerase	0.2
5 M Betaine	4
H ₂ O	7.5
Cell lysis mixture (genomic DNA template)	0.5

3. Incubate the cell lysis mixture at 55 °C with agitation overnight. The next day, inactivate the Proteinase K by an incubation at 85 °C for 1 h.
4. Prepare PCR reactions based on Table 4. PCR reaction mixtures are presented per one reaction (20 μL). Add the components listed into a DNase/RNase-free PCR tube.
5. Gently mix and briefly centrifuge to remove bubbles.
6. Place the tubes in a thermal cycler and run PCR using the following program:
 Step 1. 98 °C, 3 min.
 Step 2. 98 °C, 10 s.
 Step 3. 66–62 °C, 30 s.
 Step 4. 72 °C, 20 s.
 Step 5. Repeat steps 2–4 for 8 cycles with a 0.5 °C decrease in step 3 at each cycle.
 Step 6. 98 °C, 10 s.
 Step 7. 62 °C, 30 s.
 Step 8. 72 °C, 20 s.
 Step 9. Repeat steps 6–8 for 30 cycles.
 Step 10. 72 °C, 8 min.
7. Analyze the PCR products on 1–2% agarose gel. The expected PCR fragment sizes are oriP = 544 bp; EBNA-1 = 666 bp, and dCas9 = 1934 bp.

3.8 PCR Assembly of Guide RNA Casettes

The assembly of guide RNA cassettes consists of two parts. The first PCR reactions assemble and amplify the template DNA for the guide cassettes, and the second PCR reaction assembles the guide RNA expression cassettes from the templates and guide oligos.

1. Set up the PCR reactions for U6 promoter amplification according to Table 5 (*see Note 24*).
2. Run amplification of the U6 promoter template PCR in a thermal cycler with the following settings:
Step 1. 98 °C, 3 min.
Step 2. 98 °C, 10 s.
Step 3. 60 °C, 30 s.
Step 4. 72 °C, 9 s.
Step 5. Repeat steps 2–4 for 35 cycles.
Step 6. 72 °C, 8 min.
3. Run the PCR products on agarose gel and purify the 266 bp band with a gel extraction kit. The tailed U6 promoter amplicon sequence is presented in Fig. 4a.
4. Set up the PCR reactions for tracR template assembly according to Table 6. A schematic representation of the template oligo priming is presented in Fig. 4b.
5. Run amplification of tracR template assembly PCR in a thermal cycler with the following settings:
Step 1. 98 °C, 3 min.
Step 2. 98 °C, 10 s.
Step 3. 60 °C, 30 s.
Step 4. 72 °C, 9 s.
Step 5. Repeat steps 2–4 for 35 cycles.
Step 6. 72 °C, 8 min.

Table 5
Tailed U6 promoter template PCR

Reagent	Vol
PCR buffer 5×	4 µL
dNTP (2.5 mM)	1.6 µL
5pTailedU6promFw (10 µM)	1 µL
U6promRev (10 µM)	1 µL
Polymerase	0.2 µL
U6 template plasmid (20 ng)	x
H ₂ O	Fill to 20 µL

A Tailed U6 promoter amplicon (266 bp)

GTAAAACGACGGCCAGTgaggggcttattcccalgatccctcatattgcatacatacgatacaaggcttttagagagataattgg
aattaatttgactgtaaacacaaaagatattgtacaaaatacgtgcacgtagaaaagtaataatttctgggttagttgcgtttaaaattatgtt
ttaaaatggactatcatatgttaccgttaactgtaaaagtatttcgatttcgttgcatttatatcttGTGAAAGGACGAAACACC

B Term80 Ew

tempos EW

TermBy80hp

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ANSWER The answer is **C**. The first two terms of the sequence are 1 and 2. The third term is 1 + 2 = 3. The fourth term is 2 + 3 = 5. The fifth term is 3 + 5 = 8. The sixth term is 5 + 8 = 13.

C Tailed tracR amplicon (103 bp)

3pTailedTerm80bpRv

gttttagagctaGAAAtagcaagtaaaataaggctagtcgttatcaactgaaaaagtggaccgagtcgggcTTTTTTCATGGTCATAGCTGTTTCC

D Tailed U16 promoter amplicon

Tailed tracR amplicon



E Guide cassette assembly PCR product (455 bp)

actgaattcgatcctGAG CGTCTCACCTGTAAAACGACGGCCAGTgagggcttccatgttcataattgcatacgtata
aggcttgttagagagataattggattaattgtactgtaaacacaagatattgtacaaaatcgtgcgtagaagaataattctggtagttgc
gtttaaaattgtttaaaatggactatcatatgttccatgttgcatttgcgttatatctGTGGAAAGGACGAAAC
CCgNNNNNNNNNNNNNNNNNNNTTTTAGAGCTAGAAATAGCaaagttaaaaaggctgtccgttatcaactgtaaaaagg
gcaccgagtccgtgc TTTTTTTCATGGTCATAGTGTTCCTAACGTGAGACGagatctgtgcacgcggccgcatg

Fig. 4 Guide cassette assembly amplicons. **(a)** Sequence of the 266 bp Tailed U6 promoter amplicon used as a template for the guide cassette assembly PCR. **(b)** Priming of the Term80Fw, TermRv80bp, and 3pTailed-Term80bpRv oligos in the Tailed tracR amplicon assembly. This PCR produces the amplicon depicted in **(c)**. **(c)** Sequence of the 103 bp Tailed tracR amplicon used in the guide cassette assembly PCR. **(d)** Schematic representation of the priming of the different components used in the guide assembly PCR reaction. **(e)** Sequence of the 455 bp guide cassette assembly PCR product. Yellow marks the recognition sites of the Esp3I/BsmBI enzyme. Green marks the overhangs left by the Esp3I/BsmBI digestion. Blue marks the +1 g of the U6 promoter. This g will be included in the 5' end of the guide RNA. Red marks the site of the guide RNA sequence. Purple marks the Pol III terminator sequence at the end of the guide transcript

Table 6
Tailed tracR template assembly PCR

Reagent	Vol
PCR buffer 5×	20 µL
dNTP (2.5 mM)	8 µL
Term 80 bp Fw (100 µM)	0.5 µL
3p tailed term 80 bp Rv (100 µM)	0.5 µL
TermRv80bp (1 µM)	1 µL
Polymerase	1 µL
H ₂ O	Fill to 100 µL

Table 7
Golden Gate compatible guide RNA cassette assembly PCR from oligos

Reagent	Vol
PCR buffer 5×	20 µL
dNTP (2.5 mM)	8 µL
gRNA oligo (1 µM)	2 µL
laggc Fw (100 µM)	0.5 µL
laggc Rev (100 µM)	0.5 µL
Tailed U6 promoter amplicon (5 ng)	x
Tailed tracR template amplicon (5 ng)	x
Polymerase	1 µL
H ₂ O	Fill to 100 µL

6. Run the PCR products on agarose gel and purify the 103 bp band with a gel extraction kit. The tailed tracR template amplicon sequence is presented in Fig. 4c.
7. Prepare the PCR reaction mix for guide RNA assembly according to Table 7. Forward and reverse primers for the reaction can be matched by their numbers to produce compatible sticky ends for the Golden Gate reaction. See Table 3 for the primer sequences (*see Notes 25 and 26*). Schematic representation of the PCR reaction is presented in Fig. 4d.
8. Run guide cassette assembly PCR in a thermal cycler with the following settings:
 - Step 1. 98 °C, 3 min.
 - Step 2. 98 °C, 10 s.
 - Step 3. 52 °C, 30 s.
 - Step 4. 72 °C, 12 s.
 - Step 5. Repeat steps 2–4 for 35 cycles.
 - Step 6. 72 °C, 8 min.
9. Run 3 µL of the guide cassette assembly PCR reaction product on agarose gel to verify that the size of the guide amplicon is 455 bp. The sequence of the PCR product is presented in Fig. 4e (*see Note 27*).
10. Purify the rest of the PCR products with a PCR product purification kit (*see Note 28*).

3.9 Golden Gate Ligation of Concatenated Guide Constructs

The Golden Gate reaction uses temperature cycling between 37 and 16 °C to sequentially cut and ligate the DNA pieces together into one plasmid [20]. This reaction is used to clone the PCR-amplified guide RNA cassettes into the GG-dest plasmid backbone in a single reaction. The guides can be further cloned into the episomal plasmid backbone, e.g., GG-EBNA-OSK2M2L1-PP, with additional conventional restriction enzyme cloning step with NotI and EcoRI.

1. Prepare the Golden Gate ligation reaction mixture according to Table 8.
2. Run the Golden Gate reaction in a thermal cycler with the following settings:
Step 1. 37 °C, 2 min (restriction).
Step 2. 16 °C, 5 min (ligation).
Step 3. Repeat steps 1 and 2 for 50 cycles.
Step 4. 80 °C, 20 min (enzyme inactivation).
3. Transform chemically competent DH5α with 5 µL of the Golden Gate reaction mixture. Plate the transformed cells on LB agar plates with ampicillin. Grow the *E. coli* overnight at 37 °C.
4. Pick up and grow clones of the *E. coli* transformants overnight in LB with 50 mg/mL ampicillin.
5. Purify the plasmids with Miniprep plasmid purification kit. Verify correct clones by restriction digestion with EcoRI and

Table 8
Golden Gate guide concatenation reaction mix

Reagent	Vol (µL)
T4 ligase buffer 10×	2
T4 ligase	1
Esp3I	1
DTT (100 mM)	2
1st guide PCR amplicon (1_aggc_F/R) (50 ng/µL)	1
2nd guide PCR amplicon (2_aggc_F/R) (50 ng/µL)	1
3rd guide PCR amplicon (3_aggc_F/R) (50 ng/µL)	1
4th guide PCR amplicon (4_aggc_F/R) (50 ng/µL)	1
5th guide PCR amplicon (5_aggc_F/R) (50 ng/µL)	1
Golden gate destination plasmid (GG-dest) (150 ng/µL)	1
H ₂ O	8

NotI and sequence the plasmids to confirm correct clones from both ends with the T7 (upstream) and SP6 (downstream) primers (Table 3).

6. For cloning into the episomal plasmids, clone correct concatenated guide cassette insert from GG-dest plasmid backbone into GG-EBNA backbone (GG-EBNA-OSK2M2L1-PP) with NotI and EcoRI.

4 Notes

1. The P53 shRNA included in the plasmid is not critical, but is beneficial. Therefore, the similar plasmid without the P53 shRNA may be used: pCXLE-dCas9VP192-T2A-GFP, Addgene plasmid #, 69536.
2. The protocol described in the text uses the Neon transfection system to deliver the reprogramming plasmids into the fibroblasts. We have primarily used the Neon system to reprogram fibroblasts, but it should be possible to adapt any electroporation system that gives high efficiency of plasmid transfection to this method. The plasmid delivery system has to be efficient since the reprogramming plasmids are quite large. This protocol also includes instructions for the Countess cell counter and the Direct PCR Lysis Reagent, which our lab is routinely using. These components may be replaced with other suitable products.
3. This CRISPRa reprogramming method using episomal plasmids has been optimized with neonatal skin fibroblast cell line (HFF, CRL-2429, ATCC). We recommend setting the system up initially with a neonatal early passage fibroblast cell line to confirm that every component of the system works as expected.
4. It is advised to passage the fibroblasts at least once after thawing before reprogramming so that the cells can recover from freezing and are in good conditions. The reprogramming efficiency is generally better with actively growing early passage cells. Therefore, it is recommended to avoid keeping the cells in confluence for too long.
5. This PBS wash step removes remnants of the fibroblast culture medium that could affect the electroporation efficiency. Depending on the number of cells collected, the PBS volume can be adjusted based on the cell counting.
6. Try to aspirate all of the residual PBS from the centrifugation tube walls to prevent the electroporation resuspension Buffer R from being diluted by PBS.

7. Try to avoid introducing bubbles as this may interfere with the electroporation. Also, check that the Buffer R does not have precipitate, as this may affect the electroporation efficiency.
8. Avoid touching the electroporation tip into the fibroblast medium if a second electroporation is to be performed using the same tip.
9. Initiation of colony formation can be seen at around day 8 of reprogramming as depicted in Fig. 1e. If the starting cells for reprogramming are the adult fibroblasts or the late passage fibroblasts, emergence of colonies may take significantly longer than 8 days as presented in Fig. 1 for the control fibroblast line CRL-2429. In the case of older fibroblasts, it is recommended to keep culturing the reprogramming cells for at least 3 weeks. If colony formation is not seen after 3 weeks, the reprogramming likely does not work.
10. Tight cell clusters with small cells and defined borders can be seen at around day 12 of induction (Fig. 1f). The control fibroblast line (HFF, CRL-2429) can support iPSC culture as feeder cells in hES-medium. In the case of reprogramming other cell lines of fibroblasts, which may not function as feeders for the iPSC colonies, the cell culture medium may need to be changed to E8 Medium during the reprogramming. The time of medium switch may be when the colonies resemble those of the control day 12 colonies as shown in Fig. 1f. In the case of the adult fibroblast lines, this time point is possibly later than day 12 of induction. Cell culture medium after this point needs to be able to support iPSC growth in the absence of the reprogramming factors as the cells are expected to gradually lose the reprogramming episomal plasmids.
11. Larger colonies may need to be broken into several small pieces to prevent the clones from forming very large colonies that are prone to differentiate.
12. In the case that the colonies have not attached, add fresh E8 medium to the cells without removing the old medium. Most iPSC colonies should attach overnight but, in some cases, it may take longer.
13. Generally, it is sufficient to change the E8 medium to the iPSCs every other day. However, if the cell culture plates have high number of cells, indicated by yellowing of the pH indicator in the medium, it is good to change the E8 medium every day.
14. In larger colonies, with more compacted cells, formation of holes following the EDTA treatment may not be seen in the middle of the colonies. In this case, it is better to determine the length of the EDTA treatment based on detachment of the colony edges.

15. Generally, it is better to undertreat the early passage iPSC clones with EDTA to avoid breaking the colonies into small clumps. Early passage iPSCs do not tolerate passaging in very small clumps or single cells generated by harsh pipetting or overtreatment with EDTA. Single cells and cells in very small clumps are prone to die. The cells should adapt to passaging with EDTA within the first five passages, after which cells can be pipetted off the plates with less care.
16. Add and remove liquids carefully as described. Add 250 µL of PBS carefully from edge of the well so that you do not detach your cells. Remove PBS solution using 1-mL micropipette from edge of the well.
17. Paraformaldehyde (PFA) is toxic, use in fume hood only, and dispose of PFA in a dedicated waste container.
18. Permeabilization is only required when the antibody needs to enter inside of the cells to detect its antigen. These include intracellular proteins and transmembrane proteins whose epitopes are in the cytoplasmic region.
19. Two drops is an approximate amount of blocking solution. This is generally in the range of 100 µL. It should be sufficient to cover the cells for the duration of the blocking. The cell culture dish covers should be kept on the plates to reduce air flow around the cells and prevent the middle of the wells from drying.
20. It is critical to do extensive washing after incubation with the primary antibody to reduce nonspecific binding of the secondary antibody.
21. It is important to carry out the incubation steps in dark to prevent fluorochrome bleaching.
22. The immunoreaction is often followed by a nucleus staining with DNA dyes. Dyes such as Hoechst 33342 or DAPI can be used for this purpose. Two drops is an approximate amount and should be enough to cover the wells. Another possible approach is to add the DNA staining dye directly to the second antibody staining solution Subheading **3.6, step 10** and skip the **step 12**.
23. Successful PCR depends on template DNA integrity and purity. It is important to perform PCR reaction in DNA free environment. Clean dedicated space and pipettes with aerosol-resistant barrier tips are recommended.
24. The U6 promoter can be PCR amplified from any plasmid that has the suitable sequence. pX335-U6-Chimeric_BB-CBh-hSpCas9n(D10A) is given as an example that works with the given primer sequences. The U6 amplification forward PCR primer and the Tailed tracR/terminator reverse PCR primer

have additional tails flanking the sequences to prevent the template plasmid from amplifying in the guide assembly reaction. If this turns out to be an issue, the U6 template plasmid can be changed to some other plasmid that does not have a *Streptococcus Pyogenes* tracRNA sequence, or the promoter can be PCR amplified from the plasmid and the promoter band purified from agarose gel to reduce the template plasmid amount that may cause background amplification in the following PCR reactions.

25. The Golden Gate PCR primers aggc_F and aggc_R have long overhangs next to the Esp3I cut sites. The overhangs may possibly be shortened, but the cutting efficiency will be lower if the overhangs are too short. This can end up affecting the final Golden Gate reaction efficiency, *see* Subheading 3.9.
26. We have included OCT4 guides in Table 3 as an example for OCT4 promoter targeting with five guides. Including multiple guides per gene promoter in the guide plasmids may improve activation of the gene but it will also make the plasmid constructs larger and harder to deliver into the cells. As a rule of thumb, we recommend using two or three guides per gene promoter to ensure sufficient gene activation for reprogramming using the vectors described in this protocol. In our experience, single guide per gene may not be sufficient to support efficient gene activation for reprogramming with the activators and vectors described herein.
27. This reaction may be contaminated with another guide oligo. We recommend that you always run a water control parallel to the sample amplifications to ensure that the reaction components are clean. The water control may sometimes give a band that is smaller than the guide amplicon, but this band should not be a concern as it appears to result from nonspecific priming of the template DNA.
28. This PCR amplified guide RNA cassette can be used directly to test for guide activity by co-transfected it into cells with an activator plasmid. We have used HEK293 for this purpose. The PCR-amplified guide cassette does not transfet the cells well when used alone, but mixing it with the activator plasmids as a carrier DNA enhances its delivery.

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Chapter 13

Reprogramming Porcine Fibroblast to EPSCs

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Abstract

The development of porcine expanded potential stem cells (pEPSCs) provides an invaluable tool for investigation of porcine stem cell pluripotency and opens a venue for research in biotechnology, agriculture, and regenerative medicine. Since the derivation of pEPSC from porcine pre-implantation embryos has been demanding in resource supply and technical challenges, it is more feasible and convenient for most laboratories to derive this new type of porcine stem cells by reprogramming somatic cells. In this chapter, we describe the detailed procedures for reprogramming porcine fetal fibroblast cells to EPSC^{iPSC} with the eight reprogramming factors cloned on the piggyBac vectors followed by a selection for pluripotent cells independent of transgene expression using the EPSC media. This technique allows the generation of pEPSCs for stem cell research, genome editing, biotechnology, and agriculture.

Key words Porcine, Expanded potential stem cells, Induced pluripotent stem cell, Reprogramming

1 Introduction

The domestic pig shares extensive genetic, anatomical, and physiological similarities with humans and is considered to be an excellent model for studying human diseases, testing cell-based therapies, and to serve as a donor for xenografts [1–4]. The availability of porcine pluripotent stem cells (PSC) would be expected to substantially advance the applications of pigs in biomedical research, agriculture, biotechnologies, and regenerative medicines [5, 6]. In addition, the availability of porcine PSC would significantly facilitate the generation of transgenic animals by exploiting the abilities of pluripotent cells to self-renew for unlimited periods of time without reaching senescence. Although tremendous efforts have been made over the past few decades by many groups, the published porcine PSC lines usually do not meet the stringent criteria for pluripotency, for example, lack of robust expression of endogenous pluripotency genes and leaky expression of the exogenous reprogramming factors in the case of iPS cells. Because of low quality of those cells, they are frequently called “ES-like” or

“iPSC-like” cells [7–12]. Recently, we discovered that by modulating key molecular signaling pathways, porcine expanded potential stem cells (pEPSC) with a broad propensity for extra-embryonic and embryonic lineage differentiation potential could be derived from pig pre-implantation embryos and by reprogramming fibroblasts [13]. pEPSCs propagate robustly in culture and permit genome-editing, and thus open up many opportunities in basic research and translational applications in biotechnology, agriculture, and regenerative medicines. This chapter describes the detailed procedures for reprogramming porcine somatic cells into EPSCs (EPSC^{iPS}). The procedures described here include preparation of feeder cells, reprogramming, isolation of iPSC colonies, expansion of the established iPSC lines, freezing of the established EPSC lines, and some characterization of the lines. An important note for the generation and use of EPSC^{iPS} is that despite the many similarities between embryo-derived EPSCs and EPSC^{iPS}, differences still exist between them.

2 Materials

2.1 Preparation for STO Feeder Cells

1. STO cell.
2. Gelatin.
3. M10 medium: knockout DMEM supplemented with 10% FBS, 1× Glutamine Penicillin-Streptomycin, and 1× non-essential amino acids (NEAA).
4. 0.05% trypsin/EDTA.
5. 0.5 mg/mL Mitomycin-C solution.
6. Dimethyl sulfoxide (DMSO).
7. Freezing buffer: 90% FBS, 10% DMSO.
8. Dulbecco’s phosphate-buffered saline (DPBS) without Ca²⁺ and Mg²⁺.
9. 15-cm culture dish.
10. Tissue culture incubator.
11. Cryotube.
12. Centrifuge.

2.2 pEPSC Reprogramming and Handling

1. Mitomycin-C treated STO feeder cells.
2. Germany Landrace porcine fetal fibroblast (PFF).
3. Plasmid: PB-TRE-pOCT4-2A-pSOX2-2A-pc-MYC-pKLF4; PB-TRE-pNANOG-2A-hLIN28; PB-TRE-hRARG-2A-hLRH1; PB-CAG-rTTA; PB-EF1a-transposase. These plasmids are available from the corresponding author on request.
4. 2-Mercaptoethanol.

5. Doxycycline (Dox).
6. M15: knockout DMEM supplemented with 15% FBS, 1× Glutamine Penicillin-Streptomycin, 1× NEAA, and 110 µM 2-mercaptoethanol.
7. Reprogramming media: M15 media supplemented with 1 ng/mL human LIF, 50 µg/mL Vc, and 1 µg/mL Dox.
8. 2-Phospho-L-Ascorbic Acid (Vc).
9. Recombinant human LIF.
10. NHDF Nucleofector® Kit VPD-1001 (Lonza).
11. 10-cm culture dish.
12. pEPSC Media: For 500 mL media, 482.5 mL DMEM/F-12, 2.5 mL N2 supplement, 5 mL B27 supplement, 5 mL 1× Glutamine Penicillin-Streptomycin, 5 mL 1× NEAA, 0.1 mM 2-mercaptoethanol, 0.2 µM CHIR99021 (GSK3i), 0.3 µM WH-4-023 (SRC inhibitor), 2.5 µM XAV939, 65 µg/mL Vitamin C, 10 ng/mL LIF, 20 ng/mL ACTIVIN, and 0.3% FBS.
13. EPSC seeding media: EPSC media supplemented with 5% FBS and 10 µM Y27632.
14. 24-well plates.
15. 6-well plates.
16. Nalgene™ Mr. Frosty™ Freezing Container.
17. ROCK inhibitor Y-27632.

2.3 pEPSC-iPSC Characterization

1. RNeasy Mini Kits for standard RNA isolation.
2. QuantiTect Reverse Transcription Kit.
3. SYBR Green ROX qPCR Mastermix.
4. Primer sets for endogenous pluripotency markers RT-qPCR analysis. For OCT4, forward: 5'-tgtaccaggcttggataagttc-3', reverse: 5'-attgaacttcacccctccaacc-3'; For SOX2, forward: 5'-gtgaggggccggacagtgaactg-3', reverse: 5'-aagcgtaccgggttttctccatac-3'; for NANOG, forward: 5'-ctggaggagatctcatgattctaaag-3', reverse: 5'-agtccaggcttaagtgtctagatagaag-3'; For GAPDH, forward: 5'-ctcaacggaaagctcaactgg-3', reverse: 5'-cattgcgtacgaggaaatgagc-3'.
5. Primer sets for exogenous reprogramming factors RT-qPCR analysis. For exogenous pOCT4-2A-pSOX2-2A-pc-MYC-pKLF4 cassette expression, forward: 5'-tccaaggcagaggagaaaaag-3', reverse: 5'-ccagccaattcaagagagc-3'; For pNANOG-2A-hLIN28, forward: 5'-tgccaccagactaagctgcac-3', reverse: 5'-ctcctttgatctcgcttc-3'; For hRARG-2A-hLRH1, forward: 5'-tgcgtggaggaaatagaag-3'; reverse: 5'-gcagaggaaatggtcaggtc-3'.
6. ABI 7900 HT Sequence Detection System (Life Technologies).

7. Antibodies: anti-OCT4 (SantaCruz, SC-5279), anti-NANOG (Abcam, ab80892), anti-SOX2 (R&D, AF2018), anti-SSEA1 (STEMCELL, 60060), anti-SSEA4 (STEMCELL, 60062).
8. Staining buffer: DPBS supplemented with 1% BSA and 5% donkey serum.
9. Triton X-100.
10. DAPI.

3 Methods

3.1 Inactivation of STO Cells by Mitomycin-C and Long-Term Storage of the Inactivated Cells

Porcine EPSC^{iPS} reprogramming and cultures were routinely performed on STO feeders. To be used as feeders, the STO cells have to be inactivated. Here, we describe STO inactivation by mitomycin-C. We also describe how to store the inactivated cells for later use.

1. Coat two 15-cm dishes with 10 mL 0.1% gelatin in DPBS at 37 °C for 1 h (*see Note 1*). These dishes are referred to as gelatinized dishes. The gelatin solution will be aspirated immediately before seeding cells.
2. Take a vial of frozen uninactivated STO SNL cells (5 millions/vial) from liquid nitrogen and quickly thaw in a 37 °C water bath. Then mix the STO SNL cells with M10 media in a 1:1 ratio (1 mL cells:1 mL M10 media). Centrifuge the cells in a 15-mL tube at 300 × *g* for 5 min.
3. Suspend the uninactivated STO SNL cell pellet with M10 media and seed them to two 15-cm gelatinized dishes at a density of 2.5 millions/dish with 20 mL of the M10 media. The cells in the dishes would be confluent 3–4 days later.
4. Coat thirty-two (32) 15-cm dishes with gelatin. The coating process as described in **step 1**. After gelatin coating, aspirate gelatin solution and add 20 mL of the M10 media into each dish. Passage the uninactivated STO SNL cells from the two dishes prepared in **steps 1–3** to 32 dishes (1:16 passaging). Culture the STO cells until confluent. This usually takes 5 days.
5. Prepare mitomycin-C stock solution in DPBS at the concentration of 0.5 mg/mL (*see Note 2*). Add 360 µL of the mitomycin-C solution into each 15-cm dish. The final working concentration of mitomycin-C in M10 media is 9 µg/mL.
6. Incubate the STO cells in the mitomycin-C-containing M10 media at 37 °C, 5% CO₂ for 2.5 h. After mitomycin treatment, aspirate the media from the dishes and wash the cells once with 20 mL of DPBS. Then, add 4 mL of 0.05% trypsin/EDTA to each dish. Digest the cells at 37 °C, 5% CO₂ for 5 min.

7. Add 4 mL of M10 medium into each dish after 5-min incubation. Dissociate by pipetting, transfer the cells into a 50-mL centrifuge tube, and count the number of the cells. Centrifuge the cells at $300 \times g$ for 5 min.
8. Aspirate the medium and resuspend the cells in 20 mL of 1× freezing buffer with the cell density of 20 million cells/mL. Aliquot 0.5 mL cells into each vial and store all vials in Freezing Container. Place the container in -80°C for overnight before transferring it to liquid nitrogen tank.

3.2 pEPSC Reprogramming

The porcine EPSC^{iPS} is reprogrammed by 8 factors (*see Note 3*) (pKLF4, pOCT4, pSOX2, pc-MYC, pNANOG, hLIN28, hRARG, and hLRH) and the expression of these reprogramming factors was under the transcriptional control of the tetO2 tetracycline/doxycycline inducible promoter. All factors and rtTA-coding sequences were cloned into a piggyBac delivery vector. The expression of transposase and rtTA were driven by the human elongation factor 1 alpha (EF1a) promoter (Fig. 1a).

1. The day before electroporation (Fig. 1b) seed 4 million mitomycin-C-treated STO cells in each gelatinized 10-cm dish (*see Note 1*) and culture them with 10 mL of M10 media. On the electroporation day, aspirate the M10 media and add 10 mL of the Dox containing reprogramming media (*see Note 4*).
2. Prepare DNA for transfection: Into one 1.5-mL microcentrifuge, add the following components, 20 μL transfection buffer (VPD-1001, Lonza), 3 μg PBTRE-pOSCK + 1 μg pNhL + 1 μg hRL + 1 μg PBase + 1 μg PB-rtTA (*see Note 5*).
3. Culture pig fetal fibroblast (PFF) cells in 10-cm dishes with 10 mL of M15 media (*see Note 6*). Once the cells reach ~80% confluence, aspirate the media from the dishes and wash the cells twice with DPBS.
4. Add 2 mL of 0.05% trypsin-EDTA into one 10-cm culture dish of PFF and swirl to cover the entire surface. Digest the cells in TC incubator at 37°C for 3–5 min. Periodically, monitor the cells under microscope. Once the cells become round and begin to detach from the dish, add 2 mL of the M10 media to neutralize trypsin.
5. Dissociate cells by pipetting up and down across the dish several times. Transfer the cells into a 50 mL Falcon tube. Count cell number and transfer 1 million PFF cells into a 15-mL Falcon tube. Centrifuge the cells at $300 \times g$ for 5 min. Aspirate supernatant and resuspend the cells (1 million) with 80 μL of the Nucleofector® Kit solution (VPD-1001, Lonza) (*see Note 5*).

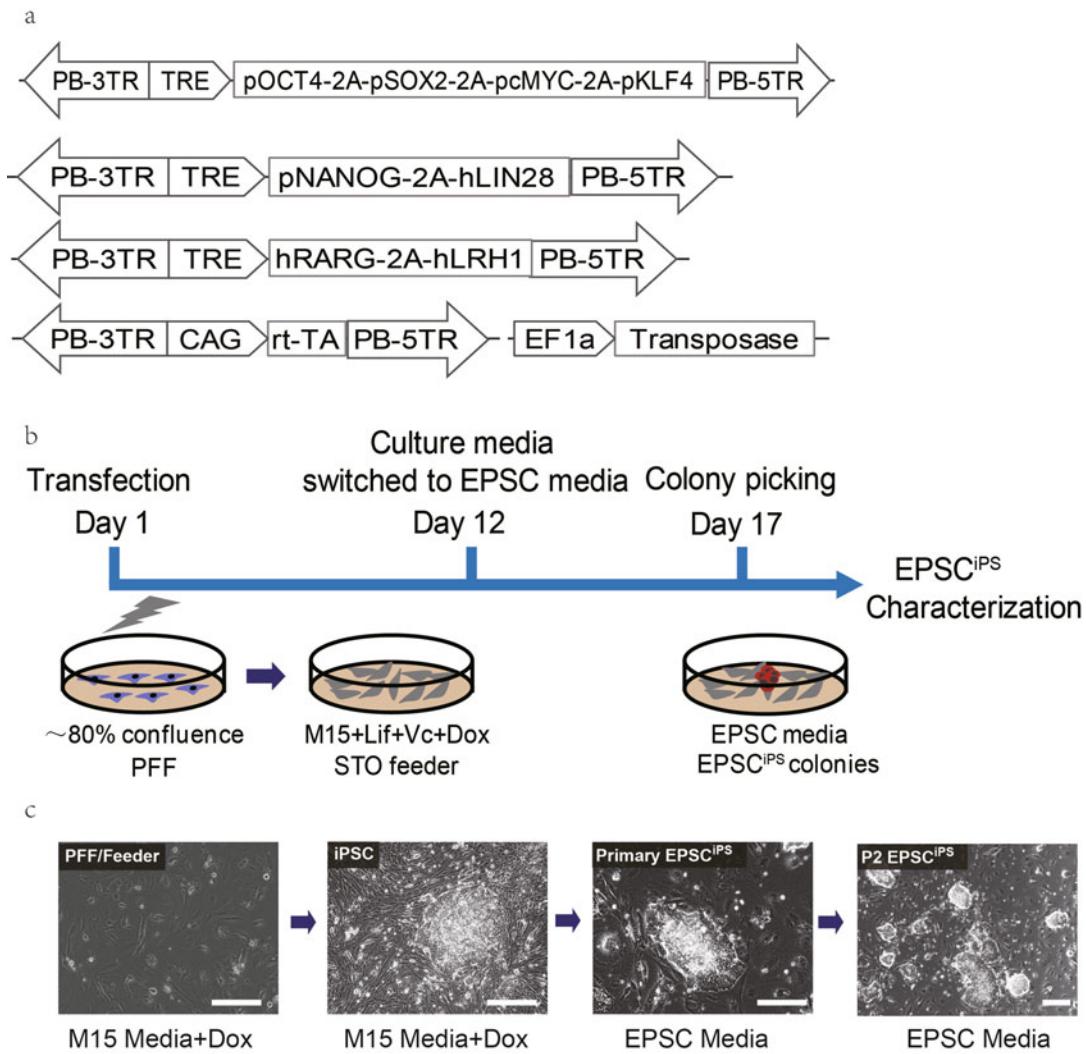


Fig. 1 Reprograming PFFs to pEPSC^{iPS}. **(a)** Four PB vectors were used: (1) Dox-inducible porcine Oct4-Sox2-Klf4-cMyc. (2) Dox-inducible porcine Nanog and human Lin28. (3) Dox-inducible human RARG and LRH1. (4) CAG-rtTA. The PB transposase was expressed transiently. Doxycycline induction started immediately after transfection. **(b)** Schematic diagram for generation of pEPSC^{iPS} from PFFs using the piggyBac (PB) transposition to deliver reprogramming factors. Timelines for treatments are indicated. **(c)** Images of porcine fibroblasts on Mitomycin-C treated STO feeder cell, iPSC colonies in reprogramming vessels, and primary pEPSC^{iPS} colony after EPSC media selection and passaged pEPSC^{iPS} colonies. Scale bars: 100 μ m

6. Transfer of the cell suspension prepared at **step 5** into the microcentrifuge tubes from **step 2** containing the DNA (*see Notes 5 and 7*).
7. Electroporate the cells by inserting the cuvette into cuvette holder. Select the program U-20. Press the “X” key to start the program. The display will show “OK” when the electroporation is complete. Add 500 μ L of the M15 media pre-warmed

at 37 °C to each cuvette and transfer 60 µL of transfected cells into each 10-cm dish containing the feeder cells as prepared in **step 1** (*see Note 8*).

8. One day later (Fig. 1c), refresh reprogramming media and then change the media every other day.

3.3 pEPSC-iPSC Colony Formation and Picking Up

1. At around day 10–12, replace the reprogramming media with the EPSC media without Dox to select the EPSC^{iPS} colonies that have become transgene independent (Fig. 1c) (*see Notes 4 and 9*). Refresh EPSC media every day and the transgene independent EPSC^{iPS} colonies would be ready for picking 5 days after media switch.
2. Three days before colonies picking, treat 24-well plate with 0.2 mL of 0.1% gelatin solution per well at 37 °C for 1 h, seed 100,000 mitomycin-C-treated STO cells in each well (*see Note 1*), and culture them with M10 media (0.2 mL/well) (*see Note 10*). On the colonies picking day, aspirate the M10 media and add 0.2 mL of EPSC seeding media into each well.
3. Pick the EPSC^{iPS} colonies (Fig. 1c) from the 10-cm reprogramming dish with a P-20 pipette tip and transfer one colony into one well of a 96-well plate filled with 50 µL EPSC seeding media. Dissociate the colony mechanically in the transit well of a 96-well plate and transfer each individual well of the cells later to one well of the 24-well feeder plate prepared in **step 2** (*see Note 11*).
4. After 24 h, switch the media to EPSC.
5. After 5 days of culture, passage to expand the picked EPSC^{iPS} colonies as instructed in the next section.

3.4 Porcine EPSC^{iPS} Expansion

1. Three days before expansion, gelatinize 6-well plates with 1 mL of 0.1% gelatin solution per well at 37 °C for 1 h. Seed 400,000 mitomycin-C-treated STO cells in each well of the gelatin-coated 6-well plate.
2. On expansion day, remove media from the EPSC^{iPS} cultured on 24-well plate and wash the cells once with DPBS. Add 100 µL 0.05% trypsin into one well of a 24-well plate and incubate the plate at 37 °C for 3 min (*see Note 12*).
3. Tap the plate on the side to detach the cells from the plate, then add fresh M10 media of equal volume to that of trypsin. Dissociate EPSC^{iPS} cells by pipetting up and down across the well.
4. Transfer cell suspension into a new 15-mL falcon tube. Centrifuge the tube at 300 × g for 5 min. Carefully remove the supernatant and resuspend the cell pellet in the EPSC seeding media.

5. Transfer the cell suspension from one well of 24-well plate to a well of 6-well new STO feeder plate. Switch the culture media to EPSC media after 24 h of culture in the EPSC seeding media. Then, change the EPSC media every day.

3.5 Porcine EPSC^{iPS} Freezing

pEPSC^{iPS} should be stored for further analyses at a later time such as characterization of the stemness.

1. When pEPSC^{iPS} colony covers 50–60% of the well surface, wash the cells with DPBS, add 0.5 mL of 0.05% trypsin for each well of a 6-well plate, and incubate for 3 min at 37 °C until pEPSC^{iPS} colonies are detached from the dish.
2. Add 0.5 mL of M10 media into each well to neutralize the digestion and to completely detach the cells by pipetting five to six times with a P1000 Pipetman. Transfer the cells into a 15-mL centrifuge tube and centrifuge the cells at 300 × g for 5 min.
3. Carefully remove the supernatant and resuspend the cell pellet in 0.5 mL of freezing buffer supplemented with 10 µM Y27632 for freezing. At this step, normally no less than 200,000 pEPSC^{iPS} cells would be harvested from one well of a 6-well plate. Store the cells from each well in one vial for freezing (200,000 cells/0.5 mL).
4. Place Freezing Container with pEPSC^{iPS} vials inside in –80 °C and transfer it to liquid nitrogen tank next day (*see Note 13*).

3.6 pEPSC^{iPS} Thawing

1. Prepare 6-well feeder plates as described in **step 1** of Subheading **3.4**.
2. Take a vial of frozen pEPSC^{iPS} cells from liquid nitrogen and quickly thaw in a 37 °C water bath. Mix the cells with 0.5 mL EPSC seeding media and centrifuge in a 15-mL tube at 300 × g for 5 min.
3. Carefully remove supernatant and resuspend cell pellet in EPSC seeding media. Then, transfer cell suspension to a well of 6-well feeder plates.
4. After 24 h of culture in the seeding media, replace the media with the EPSC media.
5. Continue to culture the cells and change the EPSC media every day until the cells reach ~60% confluence, when the cells can be passaged again or used for characterization or any of your experiments.

3.7 pEPSC^{iPS} Characterization

The quality of the established pEPSC^{iPS} should be assessed in several ways but not limited to leaking expression of exogenous reprogramming factors, and the markers are relevant to pluripotency such as OCT4, SOX2, NANOG, SSEA1, and SSEA4. The

successful reprogrammed pEPSC^{iPS} should express the endogenous pluripotency markers only, and the reprogramming factors should not be expressed upon Dox removal.

3.7.1 Expressions of Reprogramming Factors and Endogenous Pluripotency Markers in pEPSC^{iPS}

1. Total RNA of pEPSC^{iPS} was isolated using an RNeasy Mini Kit (Qiagen) for cultured pEPSC-iPSC cells. RNA was subsequently quantified and treated with gDNA WipeOut to remove genomic DNA. Complementary DNA (cDNA) was prepared using a QuantiTect Reverse Transcription Kit (Qiagen). Follow the manufacturer's instruction on above procedures step by step.
2. SYBR Green ROX qPCR Mastermix (Qiagen) were used for primer-based qPCR assays. All qPCR reactions were performed on ABI 7900 HT Sequence Detection System (Life Technologies). Follow the manufacturer's instruction on qRT-PCR procedure step by step. Representative PCR conditions: 98 °C 30 s, 40 rounds (98 °C 10 s, 58 °C 10 s, 72 °C 10 s), and 72 °C 20 s (*see Note 14*). Gene expression was determined relative to GAPDH (Fig. 2a, b).

3.7.2 Immuno-fluorescence to Confirm Endogenous Pluripotency Marker Expression in pEPSC^{iPS}

The expressions of pluripotency markers in pEPSC^{iPS} can be detected with immunofluorescence microscopy. This assay was performed directly in cell culture plate.

1. Once pEPSC^{iPS} colonies covers 20–30% of the well surface in the 24-well plate. Wash the cells twice with DPBS (1 mL/well of a 24-well plate).
2. Fix the cells with 4% paraformaldehyde (w/v) in DPBS for 10 min at room temperature.
3. Wash the cells three times with 1% BSA in DPBS for 5 min each (0.5 mL/well of a 24-well plate).
4. For staining with OCT4, SOX2, and NANOG, permeabilize and block the cells with staining buffer supplemented with 0.1% (v/v) Triton X-100. For staining with SSEA1 and SSEA4, block the cells with staining buffer only. Permeabilize and block at room temperature for 2 h (0.5 mL/well of a 24-well plate).
5. Dilute the antibodies in staining buffer to a final concentration of 3 µg/mL for antibodies. After blocking, incubate the cells with the diluted antibodies (200 µL/well of a 24-well plate) for overnight at 2–8 °C.
6. The next day, wash the cells three times with 1% BSA in DPBS for 5 min each (0.5 mL/well). At the time of washing, dilute the secondary antibody at 1:200 in DPBS supplemented with 1% BSA. For OCT4 (mouse IgG), SOX2 (Goat IgG), NANOG (Rabbit IgG), and SSEA4 (mouse IgG) (*see Note 15*), use the

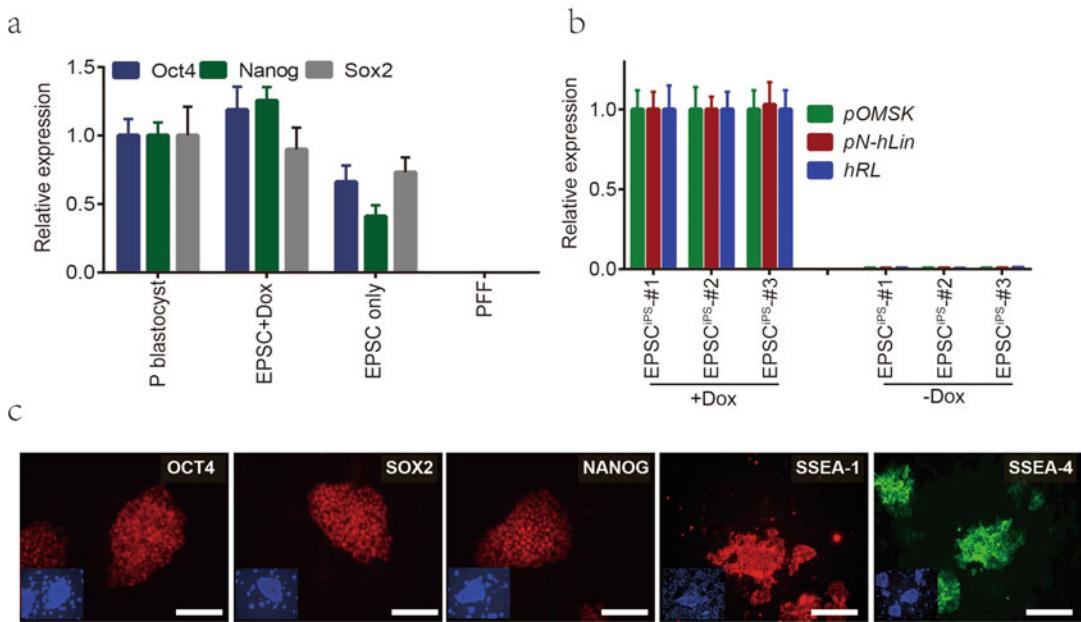


Fig. 2 Characterization of pEPSC^{iPS}. **(a)** The EPSCs^{iPS} lines expressed key endogenous pluripotency genes, as analyzed by RT-qPCR. The gene expression levels of porcine early blastocyst, PFF, and EPSCs^{iPS} cultured in Dox containing EPSC media were used as the control. The primer information is given in Subheading 2.3, item 4. **(b)** RT-qPCR analysis of the exogenous reprogramming factors in EPSCs^{iPS} either in the presence of Dox or 5 days after its removal. The primer information is given in Subheading 2.3, item 5. **(c)** Detection of pluripotency factors and the surface markers SSEA-1 and SSEA-4 in EPSCs^{iPS} by immunostaining. Scale bars: 100 μm

Alexa Fluor® 594 conjugated donkey anti-mouse/goat/rabbit IgG antibodies. For SSEA1 (mouse IgM), use the Alexa Fluor® 488 conjugated goat anti-mouse IgM antibodies.

7. Incubate the cells with the diluted secondary antibody (1:1000) in the dark for 60 min at room temperature (200 μL/well of a 24-well plate).
8. Wash the cells three times with DPBS containing 1% BSA for 5 min (500 μL/well of a 24-well plate). Incubate with DAPI in DPBS (10 μg/mL) for 10 min in dark.
9. Cells are now ready for fluorescent microscopy. Representative images are shown in Fig. 2c.

4 Notes

1. Before seeding of the uninactivated and inactivated STO cells, porcine fibroblast, and pEPSC^{iPS}. The plastic dishes should be coated with 0.1% gelatin in DPBS at 37 °C for 1 h. We used 10 mL of 0.1% gelatin solution for a 15-cm dish, 5 mL for a 15-cm dish, 1 mL for a well of a 6-well plate, and 0.2 mL for a well of a 24-well plate.

2. Shake the vials rigorously and wait for 10 min for mitomycin-C to completely dissolve. There will be some observable undissolved particles, which does not affect its potency in inactivating the fibroblasts. Make sure that mitomycin-C was added to ALL dishes. Otherwise, the whole batch cannot be used as feeders.
3. Only porcine origin of the Yamanaka factors (OCT4, SOX2, KLF4, c-MYC) produces the bona fide pEPSC^{iPS} with robust endogenous pluripotency genes expression. Human or mouse Yamanaka factors also generate the dome-shaped colonies, but these colonies have not activated the endogenous pluripotency gene network. Additional porcine origin of NANOG, combined with human origin of LIN28, RARG, and LRH1, dramatically increased the pEPSC^{iPS} reprogramming efficiency.
4. Doxycycline (Dox) was included in the reprogramming induction media to induce the expression of the reprogramming factors. Once the Dox was removed, the expressions of the exogenous reprogramming factors would be shut off. The vitamin C (2-Phospho-L-Ascorbic Acid) used in this experiments is in reduced form, and this type of vitamin C promotes reprogramming significantly.
5. The concentration of plasmid DNA stock should be between 1.0 and 5.0 $\mu\text{g}/\mu\text{L}$ and the total volume of the plasmid used for fibroblast electroporation should be less than 5 μL . Taking into account the volume of plasmids and the cell pellet, the total volume of the cell/DNA mixture in electroporation buffer would be slightly more than 100 μL , which however does not have observable effect on the transfection and reprogramming efficiencies.
6. Germany Landrace and China TAIHU PFF at the early passage proliferate quickly. These early pig fibroblasts therefore will soon take over the reprogramming dishes and push away the STO feeder cells during long reprogramming and selection processes, which leads to low reprogramming efficiency and cell detachment before colonies picking. To mitigate this issue, we normally use the PFF at passage p5-p6 to perform the pEPSC^{iPS} reprogramming experiments.
7. The electroporation should be performed without holding for long time once the cells are transferred into electroporation cuvette, as the cells in the transfection buffer would quickly sink to the bottom of cuvette. Avoiding air bubbles while transferring DNA/cell mixture from tubes to electroporation cuvette.
8. After transfection, 100,000–200,000 PFF cells were added into each 10-cm plates. The reprogramming and selection processes takes more than 2 weeks, so seeding too many cells

would make the reprogramming dish crowded and eventually affect the reprogramming efficiency and pEPSC^{iPS} colonies formation.

9. When iPSC-like colonies appear, some colonies have not activated the endogenous pluripotency genes and their proliferation and pluripotency depend on expression of the exogenous reprogramming factors. These colonies are not bona fide reprogrammed cells and cannot survive once the Dox is removed, and the medium is switched to the EPSC media. Only a small fraction (~30%) of the reprogrammed cells is true pEPSC^{iPS}, and survive in the EPSC media.
10. Feeder plates that are >4 days (4–14 days) old are ideal for seeding the picked and thawed pig pEPSC^{iPS} cells and their expansion. However, for reprogramming test, the feeder plates should be prepared just 1–2 days before fibroblast electroporation and seeding as this experiment takes 2–3 weeks.
11. When picking the single pEPSC^{iPS} colony, we would rather break the colony into small clumps rather than single cells as it helps the picked colonies to survive and recover efficiently. The passaging method with single cell dissociation can be used once those clumps form multiple descendant pEPSC^{iPS} colonies.
12. Different volumes of 0.05% trypsin-EDTA are used to dissociate the attached cells. Normally, 0.2 mL/well for a 24-well plate, 0.5 mL/well for a 6-well plate, and 2 mL for a 10-cm dish.
13. If the pEPSC in vials were stored in –80 °C for more than 10 days without transferring to liquid nitrogen, they would start to differentiate substantially after thawing for unknown reason.
14. To distinguish the expressions of the exogenous reprogramming factors and their endogenous counterparts, the primer sets for reprogramming factors were designed to amplify the fragments which cross the junction between cDNAs linked by 2A peptide coding sequence, while the primer sets for endogenous genes amplify the ones in the UTR regions, which is absent in transgenes.
15. There are barely commercial antibodies specifically designed for porcine pluripotent markers and the antibodies used in this study are for human or mouse antigens. More than two antibodies for each marker were tested and those antibodies with strongest reaction to porcine pluripotent markers are listed in Subheading 2.

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Chapter 14

Preparation of Mouse Embryonic Fibroblasts as Feeders for iPSC Generation and Maintenance

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Abstract

Mouse embryonic fibroblasts (MEFs) can be used in co-culture to support generation of induced pluripotent stem cells (iPSCs) and the normal growth and proliferation of human pluripotent stem cells (hPSCs). Here, we describe the necessary steps to derive, expand, harvest, inactivate, plate, and use MEFs as feeders for iPSC generation and maintenance.

Key words Mouse embryonic fibroblasts, MEFs, iPSC derivation, Co-culture, hPSC co-culture, Feeder cells, Inactivated MEF

1 Introduction

Co-culture techniques have long been used to offer support in in vitro mammalian cell culture. The original derivation of human embryonic stem cells (hESCs) was supported using inactivated mouse embryonic fibroblast (MEF) cells in co-culture [1]. This MEF layer contributed to the nutrient composition of the culture medium and was initially thought to be required for the undifferentiated and sustained maintenance of hESC lines in culture. Advances in media technology in the early stages of hESC research allowed the progression from direct co-culture on MEFs to the use of MEF-conditioned medium (medium placed on an MEF feeder layer for 24 h, then removed and used to culture hESCs separately) [2–4]. Early differentiation protocols also relied on the use of MEFs co-culture [5–8], as did the original derivation of human-induced pluripotent stem cells (iPSCs) [9, 10]. While later work demonstrated that human pluripotent stem cells (hPSCs) can be cultured [11, 12] and differentiated in the complete absence of MEFs [13–15], some differentiation protocols continue to rely on hPSC/MEF co-culture for maximum efficiency [16, 17]. In this

protocol, we detail the steps necessary to derive, propagate, preserve, and prepare MEFs for use in cell culture of human pluripotent stem cells. The prepared MEF here can also be used to support reprogramming of human cells into iPSCs.

2 Materials

2.1 Materials for Derivation of Mouse Embryonic Fibroblasts (MEFs)

1. Sterile instruments (dissecting scissors, iris scissors, watchmaker's forceps, assorted sharp and blunt forceps, stainless steel pans); bead sterilizer; Bunsen burner; T75 tissue culture flasks; cell counter or hemocytometer.
2. Appropriate tools to euthanize mice based on your local and federal requirements (*see Note 1*).
3. PBS without CaCl₂ and MgCl₂ (PBS−/−); 0.05% Trypsin-EDTA.
4. MEF Derivation Medium: 450 mL high glucose DMEM, 50 mL heat-inactivated fetal bovine serum (FBS) (*see Note 2*), 5 mL MEM Non-Essential Amino acid (NEAA) solution, 5 mL (10,000 U/mL) Penicillin-Streptomycin solution. Filter sterilize and store the medium at 4 °C for up to 14 days. For every mouse used in the derivation process, an average of 150 mL of MEF Derivation Medium is needed.

2.2 Materials for MEF Cryopreservation

1. Isopropanol freezing containers; cell counter or hemacytometer; 1.5 mL cryogenic vials; ice bucket and ice; −80 °C freezer.
2. PBS without CaCl₂ and MgCl₂ (PBS−/−); 0.05% trypsin-EDTA.
3. MEF Culture Medium: 450 mL high glucose DMEM, 50 mL heat-inactivated fetal bovine serum (FBS) (*see Note 2*), 5 mL MEM NEAA solution. Filter sterilize and store the medium at 4 °C for up to 14 days.
4. MEF Cryopreservation Medium: Make as needed immediately before use. To determine the needed volume, assume 0.5 mL for every vial to be frozen and add 10–20% for final volume to account for pipetting error and to achieve easier-to-work-with whole numbers. The following example is for a 25-vial freeze, scale up or down accordingly: Combine 9 mL high glucose DMEM and 3 mL heat-inactivated FBS (*see Note 2*), filter sterilize the medium. Add 3 mL of sterile DMSO (*see Note 3*), store the medium on ice until ready to use. Discard any extra medium after freezing is complete (*see Note 4*).

2.3 Materials for Thawing and Expansion of MEFs

1. T175 tissue culture-treated flasks; cell counter or hemocytometer; 6–12" metal forceps; eye personal protection equipment (PPE).

2. PBS without CaCl₂ and MgCl₂ (PBS-/-); 0.05% trypsin-EDTA.
3. MEF Culture Medium: *see item 3* of Subheading 2.2.

2.4 Materials for Passaging MEFs

1. T75 tissue culture-treated flasks; cell counter or hemocytometer.
2. PBS-/-.
3. 0.05% Trypsin-EDTA.
4. 95% ethanol.
5. MEF Culture Medium: *see item 3* of Subheading 2.2.

2.5 Materials for Inactivation and Plating of MEFs

1. Tissue culture-treated vessels (6-well plates and/or T75 flasks).
2. Sterile 0.1% gelatin solution in water.
3. Irradiation source capable of delivering 10,000 rad (100 Gy) (*see Note 5*).
4. MEF Culture Medium: *see item 3* of Subheading 2.2.

2.6 Materials for Cryopreservation of Irradiated MEFs

1. Isopropanol freezing containers; cell counter or hemocytometer; 1.5-mL cryogenic vials; ice bucket and ice; -80 °C freezer.
2. MEF Culture Medium: *see item 3* of Subheading 2.2.
3. MEF Cryopreservation Medium: *see item 4* of Subheading 2.2.

2.7 Materials for Thawing and Plating Frozen-Irradiated MEFs

1. Tissue culture-treated vessels (6-well plates and/or T75 flasks); cell counter or hemocytometer; 6–12" metal forceps.
2. Sterile 0.1% gelatin solution.
3. 95% ethanol.
4. MEF Culture Medium: *see item 3* of Subheading 2.2.

3 Methods

All reagents should be used at room temperature unless otherwise specified. Appropriate PPE, including eye protection, should be worn at all times. Review of the high-level flow chart of the protocols detailed below may be helpful to understand how each of these protocols relates to each other, and to the overall process of MEF production from derivation through use (*see Note 6*).

3.1 Derivation of MEFs

This protocol involves sacrificing mice. Any work with live animals should be performed under the strict guidance of your institution's Animal Research Committee, and in compliance with local and federal guidelines for the proper care and use of laboratory animals. This may need an approved animal user protocol. Contact your institution for guidance.

1. Obtain embryonic day E12.5 pregnant C57BL/6 mice for use in derivation of MEF cultures (*see Note 7*).
2. On day of planned derivation, prepare an assortment of sterile instruments (sharp and blunt sterile forceps, dissecting scissors, iris scissors) and sterile stainless steel pans.
3. Place the E12.5 pregnant C57BL/6 mice in a holding cage for processing.
4. Remove one mouse from holding cage, and sacrifice in accordance with all relevant institutional and federal regulations and ethical guidelines (*see Note 8*).
5. Once sacrificed (deceased), place the mouse belly up on a clean absorbent bench paper or paper towel on the benchtop. Thoroughly wet the mouse abdomen with 70% Ethanol such that no more EtOH can be absorbed. Use sterile forceps to lift the abdominal skin and dissecting scissors to cut back the skin from the lower abdomen to mid-body to expose the peritoneum (*see Note 9*).
6. Sterilize instruments between mice by washing in PBS $-/-$ to remove any tissue (*see Note 10*), submerging the tips into an ethanol bath, and carefully inserting the end into a Bunsen burner flame for 20–30 s (*see Note 11*). Place tools into a sterile pan and allow them to cool completely before using them again.
7. Using sterile dissecting scissors to cut the peritoneal wall and expose the uterine horns. Be careful not to touch the exterior of the mouse. Remove the uterine horns with forceps and dissecting scissors. Place the uterine horns into a sterile, disposable Petri dish containing about 5 mL of PBS $-/-$.
8. Sterilize all instruments as in **step 6** before moving onto the next mouse (*see Note 12*).
9. Transfer the dish containing the uterine horn into a biosafety cabinet. From this point on, use of aseptic technic is critical. Wash the uterine horn with 10 mL PBS $-/-$. Use the same wash pipet to transfer the used PBS $-/-$ to the aspiration hose (*see Note 13*). Repeat twice more. Change pipet after each rinse.
10. Cut open the uterine horn using sterile forceps and curved scissors to release the embryos (*see Note 14*). Move the embryos to one side of the Petri dish.
11. Sterilize the instruments by rinsing in PBS $-/-$, wiping off any remaining tissues, and placing the instruments into the bead sterilizer for 30 s for sterilization (*see Note 15*). Allow them to cool down completely in a sterile pan prior to reuse.

12. Wash the dish contents using a serological pipet and 10 mL of PBS $-/-$. Use the same wash pipet to transfer the used PBS $-/-$ to the aspiration hose (*see Note 13*). Repeat twice more. Change pipet after each rinse.
13. Using forceps, separate the visceral tissue from the embryos (*see Note 16*). Use forceps to transfer the embryos into a new petri dish, leaving the visceral tissue and uterine horn behind. Wipe off any attached tissue on the tools with an absorbent paper towel, wash instruments with PBS $-/-$, and sterilize them using the bead sterilizer. Count the embryos and write the number of embryos on the dish lid along with the mouse number (*see Note 17*).
14. Wash the embryos with 10 mL of PBS $-/-$ one to three times until the washing PBS $-/-$ remains clear and is not red or cloudy. While washing, carefully remove the PBS $-/-$ using a serological pipet each time to avoid aspirating tissue (*see Note 13*). If desired, embryos can be checked under a dissecting microscope (maintaining sterility) for evidence of remaining visceral tissues. If any visceral tissue becomes apparent during rinses or examination, it may be removed at this time.
15. After the final washing, remove the used PBS $-/-$ with a serological pipet (a small amount of PBS $-/-$ will remain in dish). Mince the tissues with curved scissors until the embryos are in fairly uniform, tiny (<3 mm) pieces (*see Note 18*). Rinse the tools in PBS $-/-$ and sterilize them using the bead sterilizer.
16. Add 5 mL of 0.05% trypsin-EDTA into the dish. Pipet up and down twice, but do not over pipet or the final product will be more clumpy and viscous. Place the dish in a 37 °C incubator and digest for 10 min (*see Note 19*).
17. Remove the dish from the incubator and add an additional 5 mL of 0.05% Trypsin. Pipet up and down twice to further individualize the cells (do not over pipet). Return the dish to the incubator, and incubate for additional 15 min. Trypsin digestion of cells should not exceed 25 min in total.
18. Remove the minced tissue from the incubator. Vigorously pipet the mixture up and down with a sterile 10-mL pipet until well mixed (*see Note 20*).
19. Add 10 mL of MEF Derivation Medium to the minced tissue, pipet up and down until well mixed, and transfer the contents to a sterile, 50-mL conical tube.
20. Change pipets and rinse the remaining tissue from the plate with 10 mL of MEF Derivation Medium. Transfer the rinse medium into the same 50-mL tube. Set the suspension aside at room temperature for about 5 min; this allows the undesirable clumps/chunks to settle down to the bottom.

21. To determine the number of culture flasks needed, divide the number of embryos that were minced in the dish by 3. The resulting number is the number of T75 tissue culture-treated flasks required (round-down fractions). For example: if 14 embryos were minced, 4 flasks will be used.
22. Place 10–15 mL of MEF Derivation Medium into each T75 flask. The final volume of medium and cells in the flask should be between 20 and 25 mL/T75 flask. Set aside the flasks in the biosafety cabinet.
23. Draw cell suspension from the top of the suspension using a 10-mL serological pipet and add an equal volume of the cell suspension into each prepared T75 flask. Be careful to leave cell clumps behind at the bottom of the tube (*see Note 21*).
24. Label T75 flasks appropriately, including animal number and date. The passage number is p0. Incubate the flasks containing the minced tissue mixture overnight in a 37 °C tissue culture incubator in a gas atmosphere of 5% CO₂ in air. Vented caps are excellent for this application. If using solid caps, loosen the caps one quarter turn to allow sufficient gas exchange.
25. Clean up derivation supplies and reagents and disinfect work areas (*see Note 22*).
26. The following day, remove the flasks from the incubator (tighten caps if they were loosened) and view the cultures under a microscope. Work with flasks from one mouse at a time. If there are obvious bone formations or beating cells, discard all of the flasks from that mouse.
27. If at least 90% of the flask surface of all remaining flasks is confluent with a cell layer, the cells are ready to harvest. Proceed to Subheading 3.2 for cryopreservation. The cells are usually ready for freezing within 24–48 h of plating into the flasks. If the flask surface of any remaining flasks is less than 90% confluent, aspirate the spent medium in all flasks and replace with fresh MEF Derivation Medium, and allow the cells to grow for one additional day (*see Note 23*).

3.2 Cryopreservation of MEFs

Volumes indicated are for use with T75 tissue culture flasks. If using other sizes of vessels, scale accordingly.

1. Examine the culture to determine if it is ready for harvesting. Once the cells are approximately 90% confluent, prepare to harvest the cells for cryopreservation.
2. Prepare for freezing MEFs by ensuring there are enough isopropanol freezing containers to cryopreserve the expected number of vials (*see Note 24*).
3. Work with flasks from one mouse at a time. Observe each flask under the microscope, and if any flask from a single mouse

contains contamination, bone formations, or beating cells, discard all of the flasks that originated from that mouse. Pay particular attention to flasks from embryos noted as irregular during dissection.

4. Once all flasks have been observed, work in batches of six flasks at a time (it is no longer necessary to segregate by mouse). Transfer six flasks containing cells to be harvested to the bio-safety cabinet. Keep all other flasks in the incubator until you are ready to start harvesting the next batch (*see Note 25*).
5. Because antibiotics will not be used from this point forward, take extreme care to preserve sterility (*see Note 26*).
6. Aspirate the MEF Derivation Medium from all six flasks and add 5 mL of sterile room temperature PBS-/- to each flask. Gently swirl the flasks to ensure the entire cell surface is rinsed with PBS-/- (*see Note 27*), avoid splashing the PBS-/- into the flask neck.
7. Aspirate the washing PBS-/- from all six flasks and add 2 mL of 0.05% trypsin-EDTA to each flask. Ensure that the entire cell surface is covered. Incubate 5–7 min at room temperature.
8. After 5–7 min, cap the flasks tightly and dislodge the cells by gently tapping the side of each flask with the heel of your hand three to five times. Tilt the flask from side to side to further aid in removing the cells from the culture vessel (*see Notes 26 and 28*).
9. Add 2 mL of MEF Cell Culture Medium to each flask (do not remove the 0.05% trypsin-EDTA). Use a 5-mL glass pipet to mix the cells well and to neutralize the 0.05% trypsin-EDTA. Vigorously pipet up and down in the flask to break up the cell chunks, taking care to avoid excess bubbles.
10. Pool the suspension from flasks into 50-mL conical tubes. Replace the cap on each of the flasks and set aside (they will be rinsed later).
11. Repeat collection technique for all flasks in the batch, adding cells to the same tube.
12. Using a new 5-mL pipette, return back to the first flask and add 5 mL of MEF Culture Medium, use the pipette to rinse the bottom and back of the flask to collect the remaining MEFs. Use the same pipet to transfer the same medium from flask to flask to collect additional cells. Add the collected medium to the tube containing the MEFs. Be careful not to touch the pipet to the neck of the flask during collection.
13. Continue harvesting in batches by pooling MEFs into 50-mL conical tubes. When there is 45 mL in the collection tube, move to filling a new 50-mL tube until that also reaches 45 mL. Repeat as many times as necessary (*see Note 29*).

14. Allow any large chunks to settle down in the 50-mL conical tubes (this usually takes 3–5 min). Use a pipet to transfer the supernatant from each tube into a single sterile receiver, leaving all of the chunky debris behind and creating one large pool of all of the MEFs that were harvested. Keep track of the supernatant volume as you transfer from tubes to receiver (it will be used to calculate total number of cells harvested).
15. Pool the remaining medium with chunky debris remaining in each 50-mL conical into a T75 flask and place in incubator (if there is less than 10 mL of pooled materials, add MEF Culture Medium to bring up total volume to 10 mL), and place the flask in incubator. Loosen the cap one quarter turn to vent. These plated cells will be kept in culture and used for mycoplasma and murine antibody production (MAP) testing (*see Note 30*).
16. Determine total cell number and cell density of the pooled MEFs by using a cell counter or hemacytometer. Take three individual counts of the pooled cells and average to determine cells/mL in the cell suspension (*see Note 31*). Multiply the average cells/mL by the suspension volume to determine how many cells are available for cryopreservation.
17. Calculate the number of vials that have to be frozen at 10 million cells/vial.
18. Label cryovials. Label the passage number as p1, as if it were the passage number you were using to directly re-seed the cells into a new vessel. This will be the passage number when you will re-plate the cells from your freezer. In your labels, include date, cell density, volume, and any other preferred information.
19. Unscrew the caps of 18 pre-labeled cryovials, leave caps on vials, turn on the UV in the biosafety cabinet, and sterilize the vials by UV illumination for 20 min.
20. Make MEF Cryopreservation Medium sufficient to freeze the total number of vials as determined in **step 17** (multiply total number of vials by 0.5 mL and add ~10%). Keep MEF Cryopreservation Medium on ice. Set aside an equal volume of MEF Culture Medium.
21. Evenly distribute the pooled cell suspension among 50-mL conical tubes, centrifuge at $200 \times g$ for 5 min at room temperature.
22. Aspirate the supernatant and resuspend all pellets in MEF Culture Medium (0.5 mL/vial to be frozen). Combine cells from all conical tubes into one receiver to ensure uniformity and generate a single lot.
23. Mix cell suspension thoroughly and transfer 9 mL into a sterile 50-mL tube (*see Note 32*).

24. Using a 10-mL pipette, pipette MEF Cryopreservation Medium up and down gently to ensure DMSO is properly mixed. Slowly and drop-wise add 9 mL of Cryopreservation Medium to the 50-mL tube containing 9 mL of MEF suspension. While adding the MEF Cryopreservation Medium, gently move the tube back and forth to mix the MEF cells.
25. Once all the MEF Cryopreservation Medium is added, pipette the resulting cell suspension gently, and add 1 mL of cell suspension into each prepared cryovial. Replace and tighten the vial caps, and place them in isopropanol freezing container. Immediately place the freezing container into a -80 °C freezer. Store the cells in -80 °C overnight.
26. Repeat dilution and vialing steps until all MEF cells are frozen. Be sure to thoroughly mix and resuspend the pooled cells before drawing the MEF suspension for each 18-vial batch to ensure consistency and uniformity among vials.
27. Once vialing is complete and all vials are in cold storage, clean the biosafety cabinet and remove trash (*see Note 22*, excluding carcass removal).
28. The next day, transfer the frozen MEF vials into liquid nitrogen (LN₂) storage tanks for long-term LN₂ storage.

3.3 Thawing MEFs

Do not thaw an MEF vial until negative results for mycoplasma and murine antibody production (MAP) have been received. If either test is positive, discard all vials in biohazard trash immediately and clean all containers and surfaces the materials have touched with ethanol. Volumes indicated in this section are for use with T75 tissue culture-treated flasks. If other sized vessels are used, scale appropriately. Before using for routine co-culture, each newly produced lot of MEFs should be tested alongside a proven lot of MEFs to assure it appropriately supports culture of the pluripotent stem cells (*see Note 33*).

1. Retrieve one vial of MEFs from LN₂ storage. If LN₂ storage is outside the laboratory area, transfer the vial to the laboratory in a dewar of LN₂ (or alternate transport mechanism that ensures that it remains frozen until ready to thaw).
2. Confirm that the vial cap is tightly sealed. Thaw vial by holding with a pair of 6–12" metal forceps and immersing the vial in a 37 °C water bath. Take care not to submerge the cap (*see Note 34*). Swirl the vial gently until only an ice crystal remains and remove the vial from the water bath. Immerse the vial into a 95% ethanol bath to sterilize the outside of the tube (*see Note 35*). Place the vial into the biosafety cabinet.
3. Transfer the cells gently from the vial into a sterile 50-mL conical tube using a 5 mL glass pipet.

4. Slowly add 10 mL of MEF Culture Medium drop-wise to cells in the tube. This reduces osmotic shock to the MEF cells. While adding the medium, gently move the tube back and forth to mix the MEF cell suspension and MEF Culture Medium. Centrifuge cells at $200 \times g$ for 5 min at room temperature.
5. Aspirate and discard the supernatant. Resuspend the cell pellet in 10 mL of MEF Culture Medium, pipet up and down to individualize the cells, and transfer suspension into a T75 flask (*see Note 36*). Label the flask with the passage number from the cryopreserved vial and the date (the p# on the vial should be written on the vessel).
6. Place the flask into the 37 °C incubator and position horizontally to allow the cells to attach. When placing the flask in the incubator, gently move it back and forth and side to side three times to evenly distribute the cells; avoid swirling the cells or splashing medium into the cap of the flask. If using solid caps, loosen caps one quarter turn to allow appropriate gas exchange.
7. The cells do not require medium exchange, they will grow in the same plating medium until they are ready to passage on about day 4 when they are 90–100% confluent.

3.4 Harvesting and Passaging MEFs

Volumes indicated are for use with the T75 tissue culture-treated flasks. If using T175 tissue culture-treated flasks, the appropriate volumes are indicated in parentheses in the applicable steps (*see Note 37*). If working with multiple flasks, work in batches of four to six flasks at a time.

1. Tighten caps and remove flasks containing MEFs from incubator.
2. Observe MEF cells in flasks under the microscope to ensure that there is no contamination or any other unusual situations (i.e., beating areas, large clumps, etc.).
3. Remove cap and aspirate spent MEF Culture Medium. Add 5 mL (7 mL) of PBS–/– per flask. Swirl the flask gently to wash the PBS–/– over the MEFs (*see Notes 26 and 27*).
4. Aspirate the washing PBS–/– and add 2 mL (4 mL) of 0.05% trypsin-EDTA per flask. Ensure that the entire MEF cell surface is covered. Incubate for 5–7 min at room temperature.
5. After 5–7 min, cap the flask tightly. Dislodge the MEF cells from the surface by gently tapping the side of the flask with the heel of your hand three to five times. Tilt the flask from side to side to aid in freeing the cells from the plastic (*see Note 28*).
6. Add 2 mL (4 mL) of MEF Cell Culture Medium to the flask (do not remove the 0.05% trypsin-EDTA). Use a 5- or 10-mL

glass pipet to mix the cells well and to neutralize the 0.05% trypsin-EDTA. Vigorously pipet up and down in the flask to break up the cell chunks, taking care to prevent excess bubbles from forming.

7. Use pipette to transfer the resulting suspension into a 50-mL conical tube (*see Note 32*).
8. Using a new pipette, add 5 mL (10 mL) of MEF Culture Medium to the flask. Swirl the flask to wash the surface in order to collect any remaining MEFs. Take care to avoid the liquid from entering the flask neck. Transfer the cell suspension to the tube containing the MEFs.
9. Determine volume of the MEF mixture to be seeded into flasks. Perform three cell counts using a cell counter or hemacytometer, and average to determine cells/mL. Divide 10 million cells by the resulting number of cells/mL to determine how many mLs of MEF mixture to use for subculture in a single T175 flask (*see Note 38*).
10. Hold aside the MEFs to be inactivated at this point (described below) and seed the remaining MEFs into flasks.
11. Immediately add additional MEF Culture Medium to the newly seeded flasks so that the total volume for a T175 is between 20 and 25 mL (if using T75 flasks, the final total volume is 10–12 mL/flask).
12. Label flasks, including lot number, date, technician initials, and new passage number (+1 from the previous passage number).
13. Place flasks into the 37 °C incubator and position horizontally to allow the cells to attach. When placing the flask in the incubator, gently move it back and forth and side to side three times to evenly distribute cells; avoid swirling the cells or splashing medium into the cap of the flask. If using solid caps, loosen the caps one quarter turn to allow sufficient gas exchange.
14. The cells do not require medium exchange and they will grow in the same plating medium until they are ready to be passaged on about day 3 or 4 when they are 90–100% confluent.
15. MEF cells can usually be passaged up to p6 before their quality declines as feeder cells. At that point, all the p6 cells should be harvested for inactivation (described below), and a vial frozen at an earlier passage should be thawed if a continuing supply of MEFs is needed.

3.5 Inactivation and Plating of MEFs for Use in PSC Culture

To use MEFs as a feeder layer or medium-conditioning cells for pluripotent stem cells (PSC), they must be inactivated properly (*see Note 5*) to ensure they do not continue to proliferate in culture. This protocol includes instructions for inactivating MEFs using

γ -irradiation. Use of radiation of any kind is generally overseen by an institutional Biosafety Committee and may require specific training and certification. Check with your institution to determine institutional, local, and federal regulations that apply in your area.

1. Establish a cell suspension density to be used for irradiation (*see Note 39*).
2. Prepare culture vessels by coating them with 0.1% gelatin overnight in a 37 °C incubator (*see Note 40*).
3. Dilute MEF cell suspension to appropriate density as determined in **step 1** and irradiate per institutional, local, and federal guidelines.
4. For plating MEFs to be used as a co-culture feeder, further dilute irradiated MEFs to 75,000 cells/mL using the MEF Culture Medium and mix the diluted feeder MEFs well to ensure even cell suspension. Aspirate the gelatin solution from the prepared 6-well plate (**step 2**) and plate 2.5 mL of the diluted feeder MEF cell suspension into each well of a 6-well plate (*see Note 41*).
5. When plating MEFs to be used for conditioning cell culture medium, dilute the irradiated MEFs to 210,000 cells/mL using the MEF Culture Medium (*see Note 42*) and mix the diluted conditioning MEFs well to ensure even cell suspension. Aspirate gelatin solution from the prepared T75 tissue culture-treated flask (**step 2**) and plate 20 mL of the diluted conditioning MEFs into the T75 flask.
6. Place plates (and/or flasks) with MEFs to the incubator and allow the cells to attach overnight. When placing the plate in the incubator, gently move it back and forth and side to side to evenly distribute the cells. Avoid swirling the cells. Loosen caps one quarter turn to allow sufficient gas exchange.
7. Freshly prepared irradiated MEF plates do not require medium exchange and can be maintained in the plating medium until they are used. Prior to using as a co-culture feeder, aspirate MEF Culture Medium and rinse the MEFs with PBS $-/-$ prior to use.
8. The freshly prepared irradiated MEF plates can be used as a co-culture feeder for hPSCs for up to 7 days from the day of initial plating and should not be considered supportive if the MEFs are beyond 14 days from the initial plating. The MEF prepared above can also be used for generation of human-induced pluripotent stem cells [9, 10].
9. Any extra irradiated cells not plated for culture may be cryopreserved for later use as described below.

3.6 Cryopreservation of Irradiated MEFs

Freshly irradiated MEFs may be cryopreserved for later use in varied densities to meet different needs. We routinely use densities between 5 and 20 million/vial. Do not exceed 20 million cells/vial, as recovery is compromised at higher densities. Expect to achieve a 60–80% recovery of the frozen irradiated MEFs due to the impact of freezing, irradiation, and long-term storage and cells needed for counting (depending on your counting method).

1. Determine the MEF density desired and the total number of vials that will be frozen (*see Note 43*).
2. Prepare for freezing by ensuring that there are enough isopropanol freezing containers to accommodate the expected number of vials (*see Note 24*).
3. Prepare vials, labeling with any necessary information including lot number, new passage number, freezing date, technician initials, and any additional desired information.
4. Make MEF Cryopreservation Medium sufficient to freeze the total number of vials as determined in **step 1** (multiply total number of vials by 0.5 mL and add ~10%). Keep MEF Cryopreservation Medium on ice. Set aside an equal volume of MEF Culture Medium.
5. Centrifuge the irradiated MEFs to be frozen at $200 \times g$ for 5 min at room temperature.
6. Aspirate the supernatant and resuspend pellets in a total of 0.5 mL MEF Culture Medium for every vial to be frozen (e.g., if freezing 25 vials, suspend the pellets in a total of 12.5 mL of the MEF Culture Medium). Combine all cells into one receiver to ensure the uniformity of one lot.
7. Mix cell suspension thoroughly and transfer 9 mL into a sterile 50-mL tube (*see Note 32*).
8. Unscrew the caps of 18 pre-labeled cryovials, leave caps on vials.
9. Mix MEF Cryopreservation Medium with pipet but pipetting up and down briefly. Slowly and drop-wise add 9 mL of Cryopreservation Medium to the 50-mL tube containing cells in MEF Culture Medium. While adding the medium, gently move the tube back and forth to mix the MEF cells.
10. Once all the Cryopreservation Medium is added, mix the cell suspension gently by pipetting up and down, and aliquot 1 mL/vial to each prepared cryovial. Tighten the vial caps and place in a room temperature isopropanol freezing container. Once filled, immediately place the freezing container inside a -80°C freezer and store the cells at -80°C overnight. Repeat dilution and vialing steps until all MEF cells are frozen.

After vialing each batch of 18 vials, the cell suspension needs to be thoroughly resuspended each time to ensure consistency in vials.

11. The next day, transfer the frozen MEF vials into LN₂ storage tanks for long-term LN₂ storage.

3.7 Thawing and Plating the Frozen-Irradiated MEFs

1. The day before thawing, determine the number of vials to thaw, and the anticipated number of plates that will be generated (*see Note 43*).
2. Prepare culture vessels by coating with 0.1% gelatin overnight in a 37 °C incubator.
3. Retrieve the frozen irradiated MEFs from LN₂ storage. If LN₂ storage is outside the laboratory area, transfer the vial to the laboratory in a dewar of LN₂ (or alternate transport mechanism, which ensures that the cells remain frozen until ready to thaw).
4. Confirm that the vial cap is tightly sealed. Grasp vial by the cap using a pair of 6–12" metal forceps, immerse the vial in a 37 °C water bath without submerging the cap (*see Note 34*). Swirl the vial gently. When only an ice crystal remains, remove the vial from the water bath. Immerse the vial into a 95% ethanol bath to sterilize the outside of the tube (*see Note 35*). Transfer the vial into the sterile tissue culture hood.
5. Transfer the cells gently from the vial into a sterile 15-mL conical tube using a 5-mL pipet.
6. Slowly add 10 mL of MEF Culture Medium drop-wise to the cells in the tube. While adding the medium, gently move the tube back and forth to mix the MEF cells. This reduces osmotic shock to the MEF cells.
7. Multiple vials may be thawed if necessary, and pooled prior to continuing with **step 8**.
8. Centrifuge the cells at 200 × g for 5 min at room temperature.
9. Aspirate and discard the supernatant. Resuspend the cell pellet in 0.5 mL of the MEF Culture Medium for every 1 million MEF cells thawed. Mix the MEF cells thoroughly with the glass pipet, pipetting up and down to individualize the cells.
10. Perform three cell counts using a cell counter or hemacytometer and average to determine cells/mL. Dilute the cell suspension to a final density of 95,000 cells/mL using MEF Culture Medium. Mix the cells well to ensure even cell suspension.
11. Aspirate gelatin solution from a 6-well plate and add 2.5 mL of cell suspension to each well of the 6-well plate.

12. Place the plates inside the incubator and allow the cells to attach overnight. When placing plates in the incubator, gently move them back and forth and side to side to evenly distribute cells. Avoid swirling the cells.
13. The recovered irradiated MEF cells do not require medium exchange and can be maintained in the plating medium until they are used. Prior to using as a co-culture feeder, aspirate MEF Culture Medium and rinse with PBS-/- once prior to use.
14. The plates prepared from the frozen irradiated MEFs can be used as a co-culture feeder for hPSCs up to 7 days from initial plating and should not be considered supportive if they are beyond 14 days from initial plating. The MEF prepared above can also be used for generation of human-induced pluripotent stem cells [9, 10].

4 Notes

1. In the USA, current standard is Carbon Dioxide Euthanasia Apparatus consisting of an Euthanex lid, transparent CO₂ chamber, CO₂ regulator with flow gauge, and a CO₂ supply tank. However, regulation vary by region, and you should check with your institutional Animal Care and Use Committee for information on local and federal regulations and standards in your area.
2. To heat-inactivate FBS, place an unopened, thawed bottle of FBS in a 56 °C water bath for 15 min. After 15 min, lift bottle out of water bath and gently invert and swirl the bottle. Do not shake. Place FBS bottle back in water bath for an additional 15 min. Remove FBS bottle from water bath and label the bottle appropriately to indicate that it has been heat inactivated (HI). Store the HI FBS at 2–8 °C. FBS needs to be heat inactivated once only.
3. Do not filter DMSO or any medium containing DMSO, as it will degrade the filter membrane and render the resulting medium unsterile.
4. Fresh DMSO should be used at each cryopreservation and excess DMSO should be discarded for two reasons: (1) As DMSO is exposed to air, it absorbs water and its efficacy as a cryoprotectant is reduced, and (2) Because DMSO cannot be filtered between uses, accessing the same bottle for freezing multiple lots of cells increases risk of contamination.
5. Use of radiation of any kind is generally overseen by an institutional Biosafety Committee and may require specific training and certification. Check with your institution to determine

intuitive, local, and federal regulations that apply in your area. Cesium, cobalt or a linear accelerator irradiators are the best devices for delivering a reliable amount of radiation. Alternatively, an X-ray cabinet irradiator may be used, but may deliver inconsistent irradiation doses and may not inactivate the MEFs properly. MEFs may also be inactivated with mitomycin-C, however, given the potential health risks, this option is less desirable.

6. See Fig. 1.

7. The timing of the fetal development has a tremendous impact on the ability of the resulting MEFs to support derivation and maintenance of human PSCs. We have found that embryos of E12.5 are most effective. To have the E12.5 mice ready on the day of derivation, you must work with your vendor to request

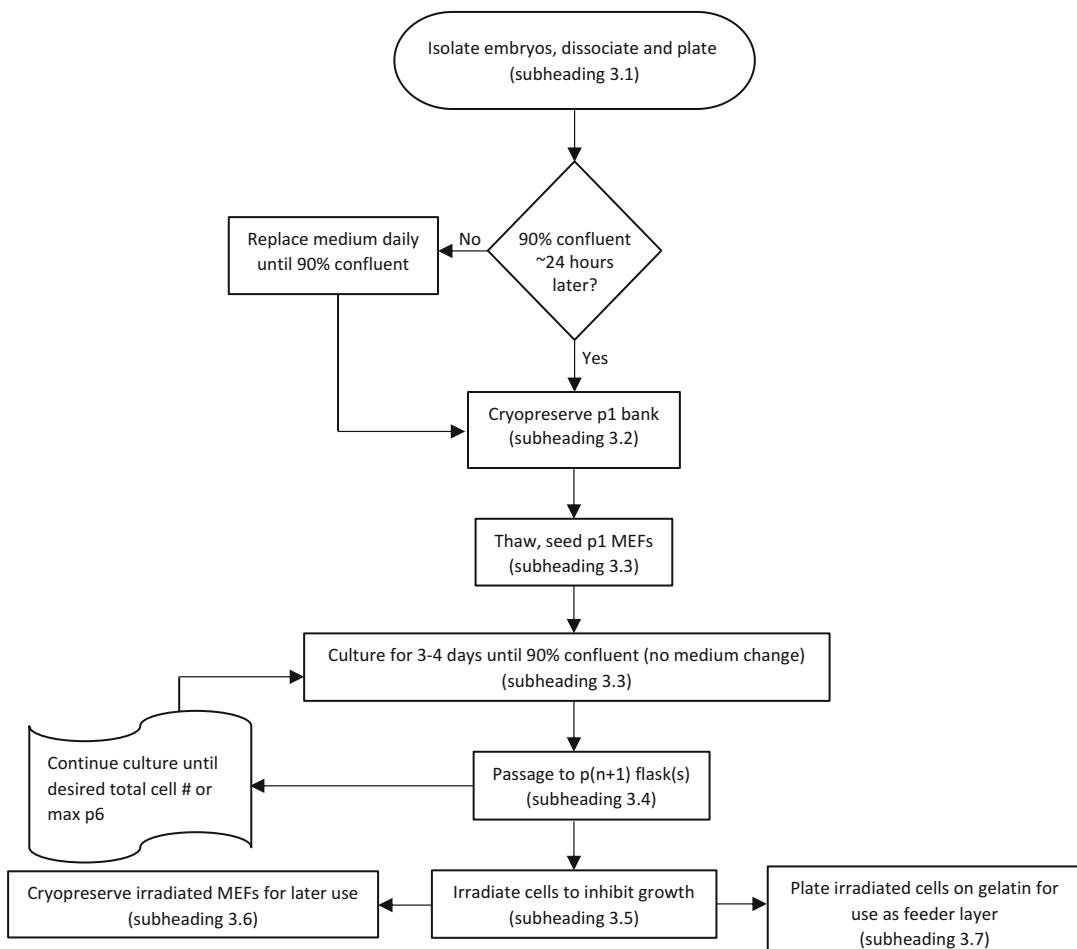


Fig. 1 High-level flow chart of the protocols included within this chapter. The flow chart details how each of these protocols relates to each other, and to the overall process of MEF production from derivation through use

specific days for breeding and delivery. We recommend contacting the vendor 6 weeks prior to any need for animals to assure their ability to provide animals appropriate for this use. Request the vendor to breed mice 13 days before your planned derivation date and request that the mice be mated as late in the day as possible. Also request that the mice arrive at your laboratory as early in the day as possible. Because of the late day breeding and early day arrival, the mice should be E12.5 pregnant on arrival. To avoid weekend work, we recommend that the initial derivation takes place on Tuesday or Wednesday to allow enough time for the whole procedure to be completed before the weekend. We have found that on average 10% of mice are not pregnant on arrival, so plan for that loss when ordering. Ordering 20 mice usually results in receiving 18 pregnant mice, which can yield up to 80–100 vials of p1 MEFs when frozen at 10 million cells/vial. If you are working in a cold climate, take possible weather-related transit delays into account during the winter months. This will avoid having the mice exposed to low temperatures for extended periods of time during transport. Mice should be processed immediately upon arrival.

8. Regulations may differ based on region. Contact your intuition for information regarding any necessary authorizations or certifications, and appropriate approved methods for your region.
9. If at all possible, instruments already used for the external dissection should not be reused for the internal dissection. A separate set of unused, clean instruments should be used for the internal dissection. If your scissors have varying degrees of sharpness, the less sharp scissors should be used for the external dissection, saving the sharper scissors for the internal dissection.
10. Having a small glass or plastic beaker (200–400 mL) with PBS−/− at the work station works well for this step.
11. Never maintain EtOH and an open flame in an enclosed environment such as a biosafety cabinet as vapors can ignite.
12. We recommend processing one mouse at a time through the entire derivation process. If working in a team, one member can be assigned to sacrificing animals, and the other of the team to processing the uterine horns once extracted. Do not sacrifice more mice at one time than can be actively processed at that time by team members.
13. To avoid aspirating tissue, use the wash pipet to transfer the used PBS−/− to the aspiration hose. Do not use a Pasteur pipet because if the needed material is accidentally aspirated, it cannot be recovered.

14. Take care to cut along the side of the horn lengthwise to avoid accidentally cutting through the embryos. The embryos may come out of the horn still inside their amniotic sac. The sac will tear easily with forceps, freeing the embryo. If any embryo appears unhealthy, too large, or remarkably unusual, discard it and do not use it in the derivation. If all the embryos in one mouse look slightly smaller or larger, make note for further examination at a later time.
15. A bead sterilizer is highly recommended, as EtOH and a Bunsen burner should never be used in an enclosed environment. Alternatively, multiple sets of sterile (autoclaved) instruments can be provided and maintained in the hood for this purpose.
16. Visceral tissue appears darker red in color than the fibroblast tissue and looks like a red dot. It is critical that all visceral tissue be removed to avoid including cardiomyocytes in the fibroblast preparation. If there are beating cells in the resulting tissue culture flask(s), all flasks obtained from that mouse will have to be discarded.
17. It may be important later in the procedure to identify which cell culture flasks resulted from which mouse. To do this, assign each mouse a number. Label all culture vessels throughout the process so that you can readily identify the mouse from which they were derived.
18. To finely mince, tip the Petri dish with one hand so that the tissue inside the dish concentrates on one side of the dish. To obtain pieces sufficiently small, you will need to mince for about 3–5 min.
19. If you are proficient in this technique, another mouse preparation can be started during this 10 min incubation.
20. This is best done by holding the pipet in a near perpendicular position to the dish and forcing the cell suspension out of the pipet. Some larger chunks are normal, but the majority of the cells should be in a fine suspension.
21. Always avoid transfer of cell/tissue clumps. Larger clumps are more likely to result in beating areas when plated, and if this happens, the entire flask with beating cardiomyocytes will have to be discarded, along with all flasks that originated from the same mouse.
22. Collect all items used for the derivation and clean/store them appropriately (tools, sterilizer, dishes, beakers, etc.). Spray down all work surfaces with a strong disinfectant such as Novalsan or Cavicide and wipe clean. Then, wipe the work surface with 70% ethanol. Place used cleaning cloths in a biohazard bag. Replace the liquid waste tube connector with a new one and place the used one in a biohazard trash bag.

Replace the nose filter in the pipet-aid with a new one and place the used one in a biohazard trash bag. Dispose of biohazard trash. Add bleach to liquid waste container as required, discard the treated liquid, and rinse the container with water. Place items that were used back into the biosafety cabinet (pens, markers, tube holders) and UV sterilize them for 20 min. Dispose of mouse carcasses and tissue appropriately per institutional and federal regulations.

23. All flasks from a derivation day should be harvested on the same day for cryopreservation. This will result in the creation of one lot of MEFs from all flasks pooled together, which will reduce testing costs. If the cell density of the flasks varies, give all the flasks one additional day to allow the sparse flasks to increase in cell numbers.
24. Replace isopropanol after every five freezes. Freezing containers must be kept in room temperature when vials are added to them.
25. Depending on the number of flasks to be harvested, it will be beneficial to have multiple people harvest to expedite the harvest process. Limiting the amount of time, the cells that are being handled will result in better viability of the resulting MEFs.
26. Strictly practice aseptic techniques. Do not allow reagents to flow down the inside of the flask neck, as this is a potential source of contamination. Likewise, avoid touching the tip of your pipette on the inside of the flask neck on its way into the flask.
27. Rinsing with PBS-/- is necessary to remove the residual culture medium that may inactivate trypsin and impair the trypsin disassociation of MEFs.
28. If working with multiple flasks, handle one flask at one time to dislodge the cells. When dislodged, set aside the dislodged flasks and continue with the remaining ones until cells have been dislodged from all flasks.
29. While a larger vessel could be used to pool the MEFs at this step, the use of the medium-sized 50-mL conical tubes allows the technician to discard a single tube of MEFs without jeopardizing the entire lot of MEFs if there are concerns regarding a potential contamination. It also allows the cell chunks to settle out and be more easily separated at when the cells are pooled.
30. Conduct testing for mycoplasma and murine antibody production (MAP) from this live culture prior to thawing any vials for further expansion. Mycoplasma may be tested for by using a PCR kit or an enzyme-based kit. External testing is usually

required for MAP. Contact your testing agency for sample submission specifications for MAP testing. Once these tests have been completed, the remaining live culture of this p0 MEFs is no longer needed and can be discarded.

31. Be sure to mix the pooled cell suspension well when obtaining sample for counting.
32. This freezing procedure is based on using freezing containers that hold 18 vials. If using freezing containers with different vial capacities, adjust the volume of cell suspension to 0.5 mL for every vial that can fit into one container, e.g., if a container holds 20 vials, transfer 10 mL of cells to a conical tube and add 10 mL of Cryopreservation Medium.
33. There is great variability among lots of MEFs. Due to this, it is strongly suggested that before using for routine co-culture, each lot of MEFs should be tested for its ability to maintain expression of the established undifferentiated PSC cell markers, and an appropriate expansion rate in PSC culture.
34. This helps reduce potential contamination that could be transferred to the vial lip by the water bath.
35. Ethanol may remove any labeling on the vial, so be sure to record any necessary information in your laboratory notebook prior to ethanol dip.
36. When thawing a lot of MEFs for the first time, one vial (at 10 million cells/vial) should be thawed into one T75 tissue culture-treated flask. If the resulting culture is too dense and needs to be passaged before day 4, the next thaw should be seeded into two T75 tissue culture-treated flasks to allow more area for expansion. MEFs that are too crowded will not proliferate well, and having appropriate thaw ratios is necessary for maximum expansion.
37. The T175 tissue culture-treated flasks can work well for routine expansion into P2 and beyond.
38. When one vial of 10 million MEF cells is thawed into one T75 tissue culture-treated flask, it typically will yield 20–30 million cells. The optimal seeding density into one T175 is 10 million (4.28×10^6 cells/cm²), however, an acceptable range is 8–11 million cells per T175 (approximately 3.4×10^6 to 4.7×10^6 cells/cm²). If only smaller T75 flasks are available, maintain the same density (3.4×10^6 to 4.7×10^6 cell/cm²).
39. In our hands, we have found both 2 and 10 million cells/mL work well when irradiated with 10,000 rad (100 Gy). Cell suspension density used in this protocol is 2 million cells/mL. To determine the appropriate density and rads that work in your circumstances, you can use this as a starting point, and dose titrate one parameter at a time, plating resulting MEFs

and ensuring that they (1) remain alive, and (2) do not further proliferate. Once optimal levels are determined, irradiate cells at a consistent density to avoid variability in outcome. Each lot of MEFs should be tested for its ability to properly support hPSC culture. To do this, we recommend culturing hPSCs with the irradiated MEFs for five passages, and assessing the resulting hPSCs to ensure they expand appropriately and continue to express the established undifferentiated PSC cell markers.

40. Use 1.5–2 mL/well of gelatin solution for a 6-well plate. If using T75 flask, add 5 mL; if using T175, add 10 mL.
41. For the traditional human PSC culture, 1.2 million MEFs are needed for six wells of a 6-well plate for use as a co-culture feeder. Alternatively, prepare a concentrated cell suspension of 1.2×10^6 cells/mL and add 1 mL to a conical tube containing 15 mL of MEF Culture Medium. This will yield the same final density and be enough to plate 6 wells of a 6-well plate at 2.5 mL/well, with extra 1 mL (to compensate for pipette error). This method is useful when preparing multiple plates. If using a different sized vessel, scale as appropriate.
42. For conditioning flasks, 4.2 million are needed for one T75 flask. Alternatively, prepare a concentrated cell suspension of 1.2×10^6 cells/mL and add 3.5 mL to a conical tube containing 16.5 mL of MEF Culture Medium. This will yield the same final density and be enough to plate one T75 flask. This method is useful when preparing many conditioning flasks at once. If using a different sized vessel, scale as appropriate.
43. Irradiated MEFs that have been frozen and thawed prior to use require a different density than freshly irradiated MEFs to support hPSC cell culture. Additionally, up to 40% of frozen irradiated MEFs will not be available for use due to death loss and cells needed for counting (depending on your counting method). We recommend a total of 1.425 million viable cells per 6-well plate if using frozen irradiated MEFs. If you assume 60% loss, it means that 2.375 million MEFs should be cryopreserved for each intended plate. For this reason, we recommend that the irradiated MEFs to be frozen be vialled in densities that are multiples of 5 million cells/mL. At 5 million cells/mL, and 1 mL/vial, one vial should produce two 6-well plates at thaw. A density of 10 million cells/vial would generate four 6-well plates, etc. We do not recommend vials at a density higher than 20 million cells/mL as viability drops sharply beyond this point.

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Chapter 15

Evaluating Reprogramming Efficiency and Pluripotency of the Established Human iPSCS by Pluripotency Markers

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Abstract

The pluripotency of human induced pluripotent stem cells (HiPSCs) cannot be tested strictly in a similar way as we can do for the mouse ones because of ethical restrictions. One common and initial approach to prove the pluripotency of an established human iPSC line is to demonstrate expression of a set of established surface and intracellular pluripotency markers. This chapter provides procedures of immunocytochemistry of the established HiPSC lines for a set of the signature intracellular pluripotency proteins, OCT4, SOX2, NANOG, and LIN28. We also describe cell phenotyping by flow cytometry for the five established human pluripotency surface markers, SSEA3, SSEA4, TRA-1-60, TRA-1-81, and TRA2-49 (ALP). Numbers of ALP⁺ and TRA-1-60⁺ colonies are the most widely used parameters for evaluation of human iPSC reprogramming efficiency. Therefore, this chapter also provides detailed steps for substrate colorimetric reaction of the ALP activity, as well as the TRA-1-60 staining, of the iPSC colonies in the reprogramming population.

Key words Immunophenotyping, Human induced pluripotent stem cells, Pluripotency, Human iPSCs, Pluripotency surface markers, Pluripotency factors, iPSC reprogramming

1 Introduction

Both mouse and human somatic cells such as fibroblasts and blood cells can be converted into pluripotent stem cells (PSCs) by ectopic expression of reprogramming factors [1–6]. Whether the induced PSCs (iPSCs) are really pluripotent or not have to be tested. The pluripotency of iPSCs can be tested in various facets [7–10]. These include: (1) expression of pluripotency surface markers [9]; (2) expression of pluripotency transcription factors; (3) expression of other genes uniquely expressed in pluripotent stem cells; (4) genome-wide profiling of the pluripotent transcriptome [7]; (5) functional test by teratoma formation [7–9]; (6) functional test by formation of the embryoid bodies; (7) functional test by directed differentiations into various lineages [9]; and (8) test of developmental potentials by injection of iPSCs into early embryos

and subsequent development in a foster mother. The last characterization is the most stringent test of pluripotency, but cannot be done for human iPSCs as for mouse ones because of ethical restrictions.

The pluripotency of the generated human iPSCs can be tested by all of the first seven approaches though. Among those characterizations, the initial and the easiest ones are expression of the pluripotency surface markers, the reigning transcriptional factors of pluripotency, and other signature pluripotency genes. Mouse iPSCs express the pluripotency surface marker SSEA1 and alkaline phosphatase (ALP). Human iPSCs, however, express five defining pluripotency surface markers SSEA3, SSEA4, TRA-1-60, TRA-1-81, and ALP, but not SSEA1 [10]. Like mouse iPSCs, human iPSCs are defined by three characteristic transcription factors, OCT4, SOX2 and NANOG. LIN28, an RNA-binding protein, is also an established pluripotency signature molecule.

In this chapter, we first describe procedures to examine the expression of the signature intracellular proteins of pluripotency, OCT4, SOX2, NANOG, and LIN28, by immunocytochemistry of the established human iPSC lines (Subheading 3.1). We then provide a protocol for immunophenotyping of human iPSCs by flow cytometry using the five established surface markers of human pluripotency, SSEA3, SSEA4, TRA-1-60, TRA-1-81, and TRA-2-49 (ALP) (Subheading 3.2). The reprogramming efficiency is commonly evaluated by the numbers of colonies positive for ALP and/or TRA-1-60 in the reprogramming vessels. Therefore, we provide additional protocols for substrate-based staining for ALP activity (Subheading 3.3), and TRA-1-60 staining of iPSCs (Subheading 3.4) in reprogramming experiments described in other chapters of this volume.

2 Materials

2.1 Reagents for iPSC Culture

1. hESC-qualified Matrigel.
2. Essential 8 (E8) Medium for human PSCs, pH 7.4, 340 mOsm [11, 12]: 1× DMEM/F-12, 1.74 g/L NaHCO₃, 13.6 µg/L sodium selenite, 64 mg/L L-ascorbic acid 2-phosphate sesquimagnesium, 10 µg/mL transferrin, 20 µg/mL insulin, 4 ng/mL basic FGF (bFGF, also known as FGF2), 2 µg/L TGFβ1 (*see Note 1*).
3. iPSC EDTA dissociation buffer: 0.5 µM ethylenediaminetetraacetic acid (EDTA), 0.18% NaCl in PBS (without calcium, without magnesium, pH 7.4). Add 1 mL of 0.5 M EDTA (pH 8.0), and 1.8 g of NaCl into 1 L of PBS (calcium-/magnesium-free, pH 7.4). Sterilize the buffer by autoclaving or by filtration with a 0.22-µM filter. Store at room temperature or at 4 °C.

2.2 Antibodies

1. Human OCT4 antibody.
2. Human SOX2 antibody.
3. Human NANOG antibody.
4. Human LIN28 antibody.
5. Goat-anti-mouse IgG1, Alexa Fluor 568 conjugated.
6. Donkey-anti-rabbit IgG, Alexa Fluor 568 conjugated.
7. PE-TRA-1-60.
8. PE-TRA-1-81.
9. PE-SSEA4.
10. PE-SSEA3.
11. PE-SSEA1.
12. PE-TRA-2-49 (ALP antibody).
13. PE-mouse IgM isotype control antibody.
14. PE-rat IgM isotype control antibody.
15. PE-mouse IgG isotype control antibody.

2.3 Equipment and Software

1. Flow cytometer.
2. Fluorescent microscope with a camera.
3. CO₂ incubator.
4. Photo scanner (Epson perfection v700 or of your choice).
5. Test-tube cooler.
6. FlowJo.

2.4 Materials for Immuno-histochemistry

1. Plastic chamber slides, 8-well (*see Note 2*).
2. PBS (without calcium and magnesium).
3. Fixation solution: 4% paraformaldehyde in PBS (pH 7.4). Dissolve 4 g of paraformaldehyde in 96 mL of PBS (pH 7.4). Put the solution inside a beaker water bath and boil the water inside a fume hood by a stirrer hot plate with gentle agitation until the solution becomes clear. Bring the volume to 100 mL with PBS. Filter the solution to remove any undissolved particles with a 0.45 µm filter. Aliquot the solution into 15-mL tubes at 5 mL each and freeze the aliquots at -20 °C.
4. Blocking buffer: 1% bovine serum albumin (BSA) in PBS with 0.3% Triton X-100. Dissolve 1 g BSA in 100 mL PBS. Add Triton X-100 at the final concentration of 0.3%, and mix well. Aliquot and store the buffers at -20 °C.
5. ProLong™ antifade mount with DAPI.

2.5 Materials for Flow Cytometry

1. Accutase.
2. 5-mL FACS tubes without caps.
3. 5-mL FACS tubes with cell strainer caps (mesh size 35 µm).
4. Cell strainers (mesh size, 70 or 100 µm).
5. FACS buffer: PBS (pH 7.4) supplemented with 2% fetal bovine serum (FBS), 1 mM EDTA, and 0.1% sodium azide.
6. 7-AAD solution: 1 mg/mL 7-Aminoactinomycin D (7-AAD). Dissolve 1 mg of 7-AAD powder in 50 µL methanol, and then add 950 µL PBS into the 7-AAD-methanol solution to achieve 1 mg/mL 7-AAD. Mix well, and then store at 4 °C. Protected from light.

2.6 Reagents for Substrate Colorimetric Staining of ALP Activity

1. ALP reaction buffer, pH 9.5: 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 5 mM MgCl₂. Dissolve 12.114 g Tris-HCl, 5.844 g NaCl, and 1.0165 g MgCl₂ in 800 mL dH₂O. Adjust pH to 9.5 and bring the volume to 1000 mL.
2. 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) stock (50 mg/mL): Dissolve 0.5 g of BCIP in 10 mL of 100% DMF. Aliquot at 1 mL per tube into 1.5 mL microcentrifuge tubes. Wrap the tube with aluminum foil to protect the BCIP from light. This stock solution is stable for 1 year at 4 °C.
3. 4-Nitro blue tetrazolium chloride (NBT) stock (50 mg/mL): First, prepare 70% N,N-dimethylformamide (DMF) as follows: add dH₂O into 70 mL of 100% DMF and bring the volume to 100 mL. Then, prepare NBT stock as follows: Dissolve 0.5 g of NBT in 10 mL 70% DMF. Aliquot at 1 mL per tube into 1.5 mL microcentrifuge tubes. Wrap the tubes with aluminum foil to protect the BCIP solution from light. This stock solution is stable for 1 year at 4 °C.
4. BCIP/NBT working solution: Add 30 µL of BCIP (0.15 mg/mL) and 60 µL of NBT (0.30 mg/mL) into 10 mL of ALP reaction buffer. The working solution should be prepared and used freshly.
5. 100% methanol, pre-cooled at -20 °C.
6. Preservation buffer for the stained iPSC colonies: PBS (calcium-free and magnesium-free) supplemented with 0.05% sodium azide.

2.7 Reagents for TRA-1-60 Staining of iPSC Colonies

1. Biotin-conjugated human TRA-1-60 antibody.
2. Streptavidin-Horseradish Peroxidase (HRP).
3. 3,3'-Diaminobenzidine (DAB) peroxidase (HRP) substrate kit (vector Laboratories, SK-4100).
4. Phosphate-buffered saline (PBS) without calcium and magnesium, pH 7.4.

5. Staining buffer: 3% fetal bovine serum (FBS), 0.3% Triton X-100 in PBS without calcium and magnesium.
6. Blocking solution: 1% bovine serum albumin (BSA) in PBS.

3 Methods

3.1 Test Pluripotency by

Immunocytochemistry for the Intracellular Pluripotency Proteins

3.1.1 Establishing Human iPSCs on Chamber Slides from the Conventional Culture Vessels for Immunocytochemistry

1. Thaw an aliquot of Matrigel inside a test-tube cooler inside a 4 °C refrigerator overnight (*see Note 3*).
2. The next day dilute the Matrigel on ice with ice-cold DMEM/F-12 to a final concentration of 75–150 µg/mL. Coat one well of a 6-well plate with 1.5 mL of diluted Matrigel at 37 °C for at least 1 h.
3. Take one vial of human iPSCs from the liquid N₂ storage tank (*see Note 4*) and thaw quickly in a 37 °C water bath. Transfer the iPSCs into a 15-mL sterile tube containing 10 mL of E8 medium pre-warmed to room temperature. Centrifuge at 400 × g for 5 min.
4. Remove the wash medium and resuspend the iPSCs in 2 mL of the E8 medium pre-warmed to room temperature supplemented with the ROCK inhibitor Y-27632 at a final concentration of 10 µM.
5. Remove the Matrigel from the well and seed the iPSCs into the coated well of a 6-well plate.
6. The next day remove the ROCK inhibitor by changing media.
7. Grow iPSCs with daily medium change.
8. When the neighboring iPSC colonies are about to merge, treat the cells with 1.5 mL of iPSC dissociation buffer at 37 °C for 5 min (*see Note 5*). Add 2 mL of the E8 medium and wash to detach the cells with a 1-mL pipette tip. Transfer the cells into a sterile 15-mL tube containing 10 mL of the E8 medium pre-warmed to 37 °C. Centrifuge at 400 × g for 5 min.
9. Remove the dissociation buffer/media from the cells and seed the cells in the E8 medium supplemented with ROCK inhibitors (10 µM) into a Matrigel coated 6-well plate (or 12-well plates, or 4-well plates).
10. The next day remove ROCK inhibitors by changing media.
11. Grow human iPSCs with daily medium change to 80% confluence in a 6-well plate (*see Note 6*).
12. Coat all the 8 wells of a chamber slide (*see Note 2*) with 200 µL per well of Matrigel pre-diluted 100× in cold DMEM/F-12 for at least 1 h at 37 °C (*see Note 7*).
13. Treat the cells with iPSC EDTA dissociation solution at 37 °C for 5 min.

14. Add 2 mL of E8 medium into the cells undergoing EDTA dissociation.
15. Use pipette tip to gently wash cells so as to detach the colonies and then transfer the cell suspension into a sterile 15-mL conical tube containing 10 mL of the E8 medium pre-warmed to 37 °C.
16. Centrifuge the cells at $300 \times g$ for 5 min.
17. Resuspend the cell pellet with 1 mL of the E8 medium.
18. Remove Matrigel from the wells of the slide chambers and add 300 µL of the pre-warmed E8 medium into each well.
19. Mix the cell suspension prepared at step 17 above. Immediately after mixing, take 30 µL of resuspended iPSCs and put the iPSCs into each well of the chamber slide. Mix gently (see Note 8).
20. Grow the cells at 37 °C, 5% CO₂.
21. The next day change the medium and make sure the cells attach to the slides as small colonies.
22. Continue to culture the iPSCs with daily change of media until the culture reaches around 80% of confluence and proceed with immunostaining as described below in Subheading 3.1.2 (see Note 9).

3.1.2 Examine Expression of the Intracellular Pluripotent Markers by Immunostaining of iPSCs Grown on Slides

1. Warm up the E8 medium, and an aliquot of fixation buffer to 37 °C (see Note 10), and the blocking buffer to room temperature.
2. Rinse the cells one time with 300 µL of the pre-warmed E8 medium.
3. Fix the cells with 200 µL of the pre-warmed fixation buffer for 15 min at room temperature.
4. Aspirate the fixation solution and rinse three times with 300 µL of room temperature PBS each time.
5. Add 200 µL of blocking buffer and incubate for 1 h at room temperature.
6. Dilute the primary antibodies in the blocking buffer according to Table 1 or based on pre-testing results if you are using antibodies from a different vendor.
7. Aspirate the blocking buffer and add 200 µL of the diluted primary antibodies.
8. Incubate overnight at 4 °C.
9. The next day aspirate the antibody solution and rinse the cells with room temperature PBS (see Note 11).
10. Wash three times with room temperature PBS for 5 min each.

Table 1
Antibodies for characterization of human iPSCs

Antibody	Brand	Cat. #	Dilution (1:X)
Anti-OCT4	Cell Signaling	2840S	200
Anti-SOX2	BD Pharmingen	561469	200
Anti-NANOG	BD Pharmingen	560109	200
Anti-LIN28	Millipore	MABD53	200
Alexa568-Goat-Anti-Mouse IgG1	Life Technologies	A11004	1000
Alexa568-Donkey-Anti-Rabbit IgG	Life Technologies	A10042	1000
PE-Anti-TRA1-60 antibody	BD Pharmingen	560193	5
PE-Anti-TRA1-81 antibody	BD Pharmingen	560161	5
PE-Anti-SSEA4 antibody	BD Pharmingen	560128	5
PE-Anti-SSEA3 antibody	BD Pharmingen	560237	5
PE-Anti-SSEA1 antibody	BD Pharmingen	560142	5
PE-Anti-ALP (TRA2-49) antibody	R&D Systems	FAB1448P	10
PE-Anti Rat IgM isotype control antibody	BD Pharmingen	553943	10

11. Dilute the secondary antibody in the blocking buffer at a 1:1000 dilution.
12. Incubate the cells in the secondary antibody solution for 1 h at room temperature, protected from light.
13. Aspirate the second antibody and rinse with PBS.
14. Wash three times with PBS at room temperature for 5 min each.
15. Remove the chambers from the slide carefully and add one drop of ProLong antifade mount containing DAPI.
16. Incubate 10 min at room temperature, protected from light.
17. Cover the sample with a coverslip.
18. Cure the sample overnight at room temperature.
19. Examine expression of the pluripotency proteins using an epifluorescence or confocal microscope. Take images (*see* Fig. 1).

3.2 Detection of Pluripotency Surface Markers by Flow Cytometry

1. Culture each cell line of iPSCs with the E8 feeder-free system in two T25 cell culture flasks until the cells reach 80% confluence in a similar way as described in Subheading 3.1.
2. When iPSCs reach 80% confluence, aspirate the medium and add 2 mL of Accutase pre-warmed to room temperature into each flask. Incubate at 37 °C for 5 min (*see* Note 12).

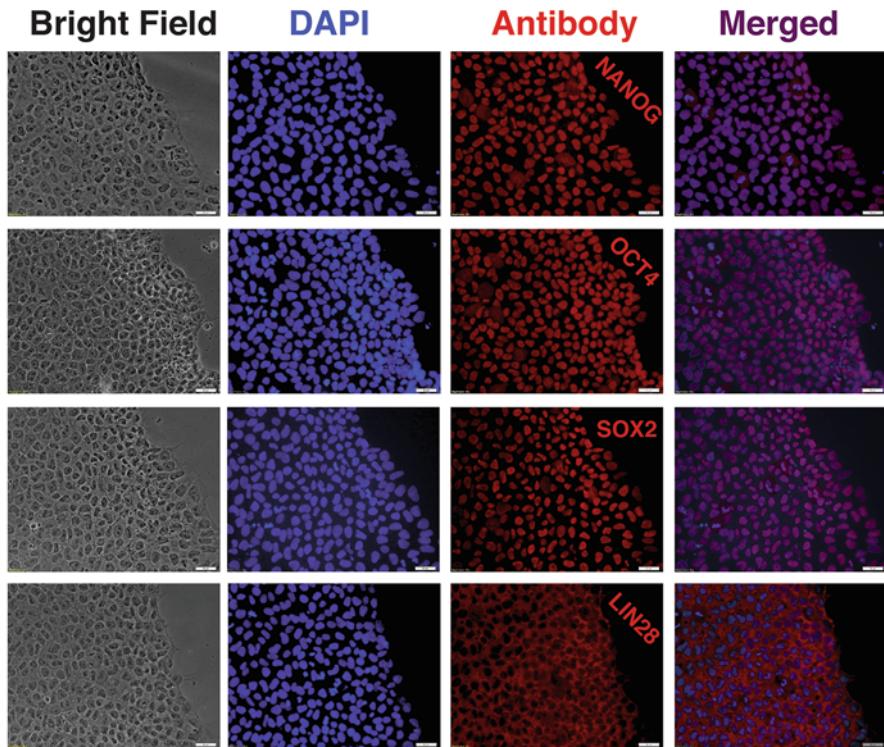


Fig. 1 Immunocytochemistry of human iPSCs. Scale, 20 μm . Images were taken at 40 \times magnitudes. Proteins stained are indicated. Nuclei were stained with DAPI

3. Pipette the cell suspension up and down to promote dissociation of cells and then transfer the cells to a 15-mL tube.
4. Add 5–10 mL of E8 medium into the tube and mix thoroughly.
5. To remove cell clumps, filter the cells vial a 70- or 100- μM cell strainer into a clean 15-mL tube (*see Note 13*).
6. Take an aliquot of 10 μL of the cell suspension and quantitate the cells using a hemacytometer.
7. Centrifuge the cells at $300 \times g$ at 4 °C for 5 min.
8. Resuspend the cell pellet in at least 1.3 mL of FACS buffer in order to obtain a cell concentration of $0.5\text{--}1 \times 10^6$ cells/100 μL ($0.5\text{--}1 \times 10^4$ cells/ μL) (*see Note 14*). Keep the cells on ice as much as possible from this step on.
9. Label the flow cytometry tubes without cell strainer caps as SSEA3, SSEA4, TRA-2-49, TRA-1-60, TRA-1-81, SSEA1, Mouse IgM, Mouse IgG, Rat IgM, TRA-2-49/No-7AAD, 7-AAD only, and No Stain (*see Note 15*). The tubes of Mouse IgM, Mouse IgG, and Rat IgM serve as the isotype controls.

10. Transfer 100 μ L of the cell suspension into each tube prepared above.
11. Add the defined amount of the PE-labeled antibodies to each tube (*see* Table 1), mix by pipetting or a quick vortex. Incubate on ice for 1 h in the dark.
12. Add 4 mL of FACS buffer to each tube and centrifuge at $300 \times g$ for 5 min at 4 °C.
13. Decant the FACS buffer containing the antibodies by inverting the tubes. Add 4 mL of FACS buffer into each tube and centrifuge at $300 \times g$ for 5 min at 4 °C.
14. Decant the FACS buffer by inverting the tubes and resuspend the iPSC pellet in 1 mL of the FACS buffer.
15. Label the flow cytometry tubes with cell strainer caps (mesh size, 35 μ m) the same way as in **step 9** of this section.
16. In order to remove any cell clump, filter the cell suspension by transferring the cells into new flow cytometry test tubes through the cap cell strainer labeled above (*see* Note 16).
17. Add 7-AAD solution to a final concentration of 0.5 μ g/mL and incubate for 5 min on ice before analysis with a flow cytometer.
18. Conduct cell immunophenotyping using a flow cytometer and acquire at least 10,000 cell events for each sample (*see* Note 17).
19. Analyze the data using FlowJo (Fig. 2).

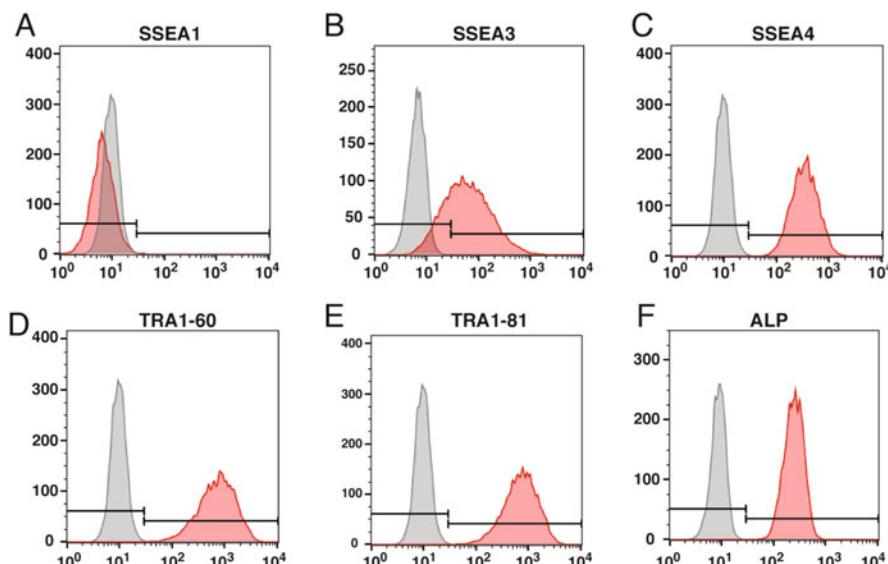


Fig. 2 Immunophenotyping of human iPSCs using the five pluripotency surface markers as indicated. SSEA1 is used as the negative control. Grey histogram is isotype control; and pink is antibodies as indicated

3.3 Evaluate Human iPSC Reprogramming Efficiency by the Number of the Colonies Positive for Alkaline Phosphatase

The efficiency of human iPSC reprogramming is commonly evaluated by the numbers of the colonies positive for alkaline phosphatase. The starting materials for this section is the reprogramming cells from day 8 to day 30 described in some of the other chapters of this volume depending on the users' motivation for this evaluation. This protocol can also be used to stain the iPSCs of the established iPSC lines.

1. Aspirate the media from the reprogramming cells or the established iPSCs and wash the cells with PBS once (1 mL for one well of a 12-well plate, 2 mL for each well of a 6-well plate).
2. Add 100% methanol pre-cooled to -20°C into each well (1 mL for one well of a 12-well plate, 2 mL for each well of a 6-well plate) and incubate at RT for 10 min (*see Note 18*).
3. When fixation is going on, prepare the working BCIP/NBT solution using the stock BCIP, NBT solutions, and the ALP reaction buffer (*see Subheading 2*).
4. Aspirate the methanol and rinse the cells with PBS two times.
5. Rinse the cells with the ALP reaction buffer once.
6. Add BCIP/NBT solution (1 mL for one well of a 12-well plate, 2 mL for each well of a 6-well plate) and incubate for 15 to 25 min in the dark at RT. Check the color intensity periodically to decide when to stop the colorimetric reaction.
7. After the incubation, aspirate the BCIP/NBT solution and add PBS containing 0.05% sodium azide solution (1 mL for one well of a 12-well plate, 2 mL for each well of a 6-well plate) (*see Note 19*).
8. Count number of the ALP^{+} colonies for each treatment of reprogramming and calculate reprogramming efficiency based on the number of the starting cells.
9. Scan the wells of the stained iPSC colonies using a photo scanner or take pictures with a microscope (*see Note 20*) (Fig. 3).

3.4 Evaluate Human iPSC Reprogramming Efficiency by the Number of Colonies Positive for TRA-1-60

The efficiency of human iPSC reprogramming is also evaluated by the number of the colonies positive for TRA-1-60. The starting materials for this section is the reprogramming cells from day 8 up to day 30 described in some of the other chapters of this volume depending on the purpose of this evaluation. It can be used to stain iPSCs of the established cell lines as well.

1. Aspirate the culture medium from the 6-well plate (*see Note 21*) and rinse the wells twice with 2 mL of 1× PBS for each well. After the last rinse, add 2 mL of 100% methanol pre-cooled to -20°C and fix at room temperature for 10 min (*see Note 22*).

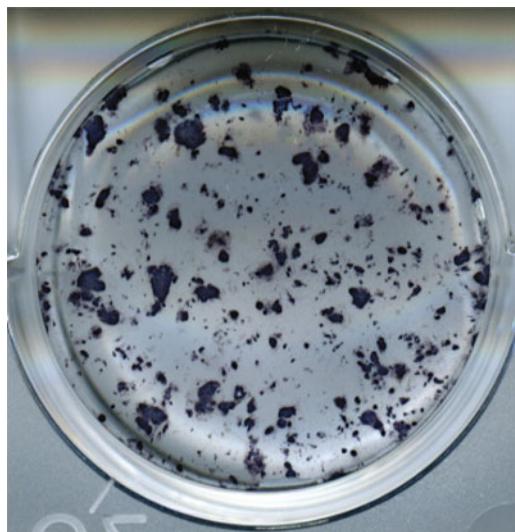


Fig. 3 A representative scanned image of reprogramming cells stained for the ALP activity

2. Remove the fixative from the wells and rinse the cells twice with 2 mL of 1× PBS each time.
3. To reduce the background, block the cells with 1% BSA in PBS at room temperature for 30 min. After blocking, rinse the cells twice with 2 mL of 1× PBS each time.
4. Dilute the biotin-conjugated TRA-1-60 antibody (1:200) in 1.5 mL of the staining buffer and add the diluted antibody into one well of a 6-well plate. Incubate overnight at 4 °C.
5. Aspirate the primary antibody and wash the cells three times with 2 mL of 1× PBS for 2 min each time with very gentle agitation.
6. Dilute streptavidin-HRP-conjugated secondary antibody (1:500) in the staining buffer. Aspirate PBS and add 1.5 mL of the diluted secondary antibody into each well. Incubate at room temperature in the dark for 2 h.
7. Remove the diluted secondary antibody reagent and wash the wells five times with 2 mL of 1× PBS for 2 min each time with very gentle agitation.
8. Prepare the substrate working solution from the DAB HRP kit: to 5 mL of water, add two drops of reagent 1, four drops of reagent 2, two drops of reagent 3, and two drops of the nickel solution. Add 1.5 mL of the substrate working solution into each well and incubate at room temperature for 10–15 min.

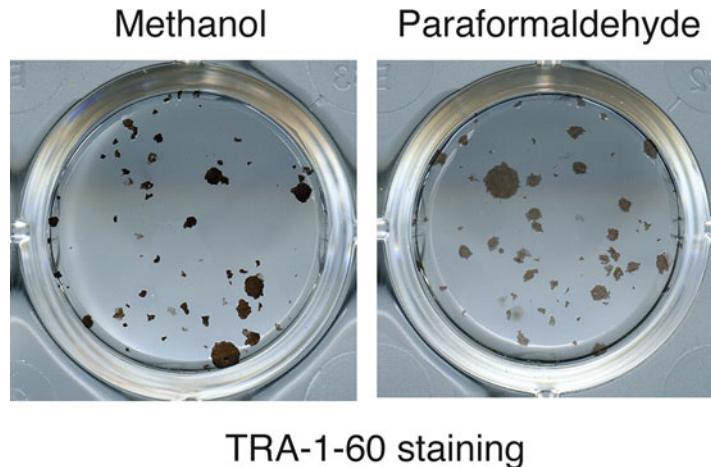


Fig. 4 Representative scanned images of the reprogramming cells stained for TRA-1-60. Please note the more intense staining for colonies fixed with methanol than that with paraformaldehyde

9. Remove the substrate and rinse the cells three times with 2 mL of 1× PBS each time. Add 2 mL of preservation buffer into each well and store the cells at 4 °C if analysis cannot be conducted right away (*see Note 19*).
10. Count the TRA-1-60⁺ colonies. Calculate the reprogramming efficiency.
11. Take images using photo scanner and/or microscope (Fig. 4) (*see Note 20*).

4 Notes

1. The E8 medium for the maintenance of human iPSCs and embryonic stem cells are available commercially if the users of the protocol prefer not to make it by themselves.
2. We use plastic slide chambers because iPSCs attach much better to plastic slides than to glass slides.
3. Genetically modified vitronectin supporting growth of human pluripotent stem cells are available commercially [11]. Follow the protocol provided by the manufacturer if the users choose to use hESC-qualified vitronectin for coating.
4. When growing iPSC culture is available, directly start from **step 12** of Subheading 3.1.1 to transfer iPSC culture from the conventional culture vessels into slide chambers.

5. Instead of the iPSC dissociation buffer prepared in-house, we found that the commercial enzyme-free Gentle Dissociation Reagent (Stemcell Technologies, Cat#, 07174) works well.
6. Immunocytochemistry can be conducted for cells grown on conventional cell culture vessels. Go to Subheading 3.1.2 directly if you prefer to conduct immunocytochemistry on 6-well or 12-well plates. Please note that you have to modify Subheading 3.1.2 slightly because it is written specifically for immunocytochemistry of cells on a slide. You will have high-quality images of stained cells when slides are used, especially for images of high magnification (20 \times and 40 \times , or higher).
7. Coating the slides with the concentrated Matrigel promotes attachment of iPSCs to slides (dilution factor of 100 vs around 1,000 in the conventional passaging of iPSCs.).
8. Unlike the conventional passaging of iPSCs with E8 system, we do not use ROCK inhibitors when passaging iPSCs onto slide chambers. ROCK inhibitors trigger differentiation of iPSCs. Omission of ROCK inhibitors will ensure high quality of iPSCs for the subsequent marker staining. iPSCs attach well even without the use of ROCK inhibitors when we use concentrated Matrigel to coat the chamber (dilution factor of 100 vs around 1,000 in the conventional passaging of iPSCs.).
9. Keep cultures below 90% confluence to avoid spontaneous differentiation.
10. Cold wash media and fixation solution loosen the attachment of iPSC colonies to the slides, and the quality of the subsequent imaging is affected because of difficulty in focusing the entire colony. We found that warming the solutions and media to 37 °C removes this issue, and high-quality images can be achieved.
11. Add the PBS gently during washes and avoid the cells and antibodies from drying to preserve antibody integrity from this step on.
12. Using Accutase instead of trypsin to dissociate iPSCs into individual cells reduces the possible enzymatic digestion of your cell surface proteins and renders higher viability of the treated cells after cell dissociation.
13. This filtration step is optional since step 16 will filter the cells again immediately before analysis with flow cytometry.
14. Adjust the volume appropriately considering that you will need at least 12 tubes per cell line. It is expected that you will get around 8–10 \times 10⁶ cells from two T25-flask cultures at 90% of confluence, which is enough to have more than 5 \times 10⁵ cells per tube.

15. The expression of ALP is generally high and uniform in pluripotent stem cells, giving strong signals and a narrow bell-shaped histogram. Therefore, ALP/TRA-2-49 is the chosen marker for compensation in the flow cytometry analysis. The TRA-2-49/No-7AAD tube is for compensation to set up the flow cytometer.
16. Cells do not flow through the strainer efficiently by gravity. Pipette up and down the cell suspension in the reservoir of the cell strainer to facilitate the filtration using a 1-mL pipette tip.
17. It is encouraged to conduct flow cytometry immediately after the staining. Cells should be stored on ice to prevent the cells from death if flow cytometry cannot be conducted immediately. Cells can stay on ice for 4 h before analysis. Prolonged storage of the cells after staining will result in significant cell death.
18. Do not fix the cells with paraformaldehyde because it will compromise the enzyme activity of alkaline phosphatase. To preserve the ALP activity, it is critical to fix the iPS cells with methanol, which is much milder. In our experience, ALP staining becomes very weak after fixation with paraformaldehyde, but methanol fixation preserves the ALP activity and gives intense signals.
19. At this stage, the stained plates can be stored at 4 °C for later analysis. Inclusion of sodium azide prevents growth of microbes.
20. During scanning of the stained cells, the preservation buffer should be removed to improve quality of the images. The reflective liquid in the wells will result in glare in the images.
21. This protocol is written for procedures with a well of a 6-well plate. The amount of reagents can be adjusted depending on the size of your reprogramming vessels.
22. Some published methods used 4% paraformaldehyde to fix the cells for TRA-1-60 staining [13]. We found that like staining for ALP activity, paraformaldehyde significantly damages the TRA-1-60 epitope and methanol fixation gives much stronger staining (Fig. 4).

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Chapter 16

G-Banded Karyotyping of Human Pluripotent Stem Cell Cultures

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Abstract

Acquired chromosomal abnormalities may occur during the reprogramming and culture of human pluripotent stem cells (hPSCs). Therefore, it is required that regular testing of genetic integrity be conducted. G-banded karyotyping is a widely used genetic assay that is capable of detecting chromosomal abnormalities. Karyotyping of hPSC cultures can be a challenging undertaking for inexperienced investigators; here, we provide detailed procedures for karyotyping, including sample preparation and analysis, as well as the interpretation of hPSC karyotype results.

Key words G-banded karyotyping, Human pluripotent stem cells, Cytogenomics, Genetic stability testing, Recurrent acquired karyotypic abnormalities

1 Introduction

The reprogramming required for the derivation of human pluripotent stem cells and the subsequent routine culture can permit or induce the acquisition of genetic abnormalities in the resulting cells [1]. Certain abnormalities are reported to be recurrent (nonrandom) and the aberrant cell population may overtake the cultures because of a growth advantage over the wild type cells [2–9]. The altered hPSC genomes can undermine research goals by producing confounding results and affecting the reproducibility of the work [10]. Furthermore, abnormal hPSCs share similarities to cancer cells, including apoptotic resistance [11] and reduced differentiation potential [12]. Therefore, routine testing of hPSC cultures is recommended to ensure the genetic integrity of the cell cultures [13], typically every 5–10 passages [10]. G-(Giemsa) banded karyotyping is a cytogenetic assay commonly employed in both clinical and research settings. Karyotyping evaluates the entire genome at a resolution of ≥ 5 –10 Mb [14] and can detect abnormal subpopulations as low as 14% mosaicism [15] assuming a standard analysis [16–18] of twenty cells. Although submicroscopic variants beyond

karyotype resolution can occur [2], karyotyping remains a versatile assay, especially when complemented with high-resolution testing [19]. Here, we describe the detailed procedures of hPSC karyotyping including initial specimen accessioning, cell harvest, slide preparation, chromosome staining, chromosome analysis, and interpretation of the results.

2 Materials

Review the Safety Data Sheet (SDS) of any unfamiliar material before handling. Wear personal protective equipment (PPE) to reduce exposure to materials.

2.1 Accessioning and Cell Harvest

1. Ethidium bromide working solution (0.1%): Ethidium bromide is a potential carcinogen and mutagen. Prepare in the biosafety cabinet by diluting 1% ethidium bromide stock solution by 1:10 in sterile water. Aliquot quantities of 1 mL each into 1.5 mL tubes and store the aliquots at 2–8 °C. Protect from light by wrapping each tube with aluminum foil.
2. 1× Trypsin-EDTA working solution (0.05%): Prepare in the biosafety cabinet by diluting 10× trypsin-EDTA by 1:10 in Hank's Balanced Salt Solution. Aliquot volumes of 6 mL each into 15 mL centrifuge tubes and freeze the aliquots at –20 °C.
3. Trypsin stop solution (10% FBS): Prepare in a biosafety cabinet by diluting 50 mL of FBS in 450 mL DMEM-F12. Aliquot quantities of 5 mL each into 15 mL tubes and freeze the aliquots at –20 °C.
4. Modified Carnoy's fixative: Modified Carnoy's fixative is a flammable and volatile solution. It can form high vapor concentrations at room temperature and is toxic if ingested; prepare the reagent in a fume hood immediately before using. Add one part glacial acetic acid to three parts 99.9% methanol.
5. Colcemid (10 µg/mL): Store at 4 °C.
6. Hypotonic solution (0.075 M KCl): Store at 4 °C.

2.2 Slide Preparation

1. Cytogenetic drying chamber (Thermotron).
2. Modified Carnoy's fixative: the same as item 4 of Subheading 2.1.

2.3 Chromosome Staining

1. Stock Leishman's stain solution: Methanol and the Leishman's stain solution (containing methanol) are flammable and volatile solutions. High vapor concentrations form at room temperature, and it is toxic if ingested; prepare the reagent in a fume hood. Dissolve 1 g of Leishman's stain with 500 mL of 99.9% methanol in a 1 L sterile bottle and completely wrap the bottle in aluminum foil. Place the bottle on a stir plate and mix

overnight with a magnetic stir bar. Filter the stain solution into a new 1 L sterile bottle using a filter funnel with a #1 Whatman filter. Filter a second time into an amber glass bottle using a new filter. Age at room temperature (protected from light) for a minimum of 2 days before use. Store in a dark cabinet for up to 1 month but discard the solution if precipitates appear.

2. Gurr buffer: Add 1 Gurr buffer tablet (pH 6.8) into 1 L of distilled water in a 1 L sterile bottle. Place the bottle on a stir plate and mix the buffer with a magnetic stir bar for 2 h or until tablet is completely dissolved; avoid over-stirring. Store at room temperature for up to 6 months.
3. 1× Trypsin banding solution: Add 5 mL of 10× trypsin-EDTA to 45 mL of Hank's Balanced Salt Solution in a Coplin jar.
4. Working stain solution: Add 11 mL of the stock Leishman's stain solution to 33 mL of Gurr buffer in a Coplin jar. Oxidized stain (shiny appearance) may form on the surface after a few minutes; skim oxidized stain off surface using a laboratory tissue. Agitate the working stain solution prior to use if it has been sitting for more than 10 min and discard the working stain solution after 4 h.
5. Cytoseal 60.

2.4 Chromosome Analysis

1. Brightfield microscope.
2. 10× objective.
3. 60× or 100× objective.
4. Charged Couple Device (CCD) camera.
5. Acquisition and analysis software.
6. Immersion oil.
7. Optical lens cleaner.

2.5 Data Interpretation

1. Analysis and reporting software.

3 Methods

Observe aseptic techniques and universal precautions throughout the procedure. Use a new pipette for each specimen and replace tip if it touches the cells, culture media, or the sides of the culture vessel or sample tube.

3.1 Specimen Accessioning and Cell Harvest

1. Upon receipt, designate the cell culture with a unique identifier and record cell culture information (*see Note 1*). Ensure that the cell culture is ready for harvest (*see Note 2*).

2. Prior to cell harvest, pre-warm the 1× trypsin-EDTA, trypsin stop solution, and hypotonic solution to 37 °C (*see Note 3*).
3. Transfer the culture vessel to the biosafety cabinet and add 10 µL ethidium bromide working solution per 1 mL of media, and gently swirl (*see Note 4*). The final concentration is 0.001% (10 µg/mL). Place the culture vessel in a 37 °C incubator and incubate for 38 min.
4. Move the culture vessel from the incubator to the biosafety cabinet and add 20 µL of 10 µg/mL Colcemid (stock solution) per 1 mL of media, and gently swirl (*see Note 4*). The final concentration is 200 ng/mL. Return the culture vessel to the 37 °C incubator and incubate for 30 min.
5. Detach the cells from the vessel (*see Note 5*). Transfer the culture vessel to the biosafety cabinet, aspirate the media from the culture vessel, and add the pre-warmed 1× trypsin-EDTA working solution. Place the culture vessel in a 37 °C incubator and incubate for 5 min.
6. Return the culture vessel to the biosafety cabinet and pipette the working solution gently up and down over the detaching cells (*see Note 6*). Pool the detached cells in working solution into one 15 mL centrifuge tube containing trypsin stop solution, then centrifuge for 5 min at 200 × *g*.
7. Expose the cells to the hypotonic solution by first transferring the centrifuge tube to the biosafety cabinet and aspirating the supernatant to just above the cell pellet. Resuspend the pellet by tapping the bottom of the tube. Add 5 mL of warmed hypotonic solution dropwise with a sterile serological pipette while manually agitating the tube (*see Note 7*). Incubate the tube in a 37 °C water bath for 18–25 min.
8. Transfer the tube from the water bath to the fume hood and gently tap the tube to resuspend the cell pellet in the hypotonic solution. Add 20–25 drops of fixative to the cell suspension (*see Note 8*). Mix the contents by gently inverting the tube and incubate at room temperature for 5 min. Then centrifuge the tube for 5 min at 200 × *g*.
9. Aspirate the supernatant to just above the cell pellet in the fume hood and tap the tube to resuspend the cell pellet. Add 5 mL of fixative and incubate at room temperature for 30 min (“first fix,” *see Note 9*). Following incubation, centrifuge the tube for 5 min at 200 × *g*. Repeat this step again for a “third fix.”
10. Transfer the sample back into the fume hood. Aspirate the supernatant and tap the tube to resuspend the cell pellet. Add 5 mL of fixative (“second fix”), then centrifuge the tube for 5 min at 200 × *g*. Repeat this step again for a “third fix.”

11. Transfer the sample back into the fume hood. Aspirate the supernatant and tap the tube to resuspend the cell pellet. Add fixative very slowly until the optimal cell suspension is achieved for the preparation of metaphase chromosome spreads (*see Notes 9 and 10*). The sample is now in “final fix.”

3.2 Slide Preparation

1. Label slides with sample identifiers and store in 100% ethanol prior to use.
2. Set cytogenetic drying chamber to 24 °C and 33% humidity (*see Note 11*).
3. Within the cytogenetic drying chamber, immerse a microscope glass slide in a Coplin jar containing distilled water until the residual ethanol is washed off.
4. Clean off the slide with a laboratory tissue, and reimmerse the slide in distilled water. Allow the water to drain from the slide so that a thin film of water remains.
5. Resuspend the cell pellet using the plastic transfer pipette. Starting at the frosted end, drop 6–10 drops of the cell suspension along the length of the slide. Hold the slide flat while dropping the cell suspension.
6. Immediately flood the slide with one full Pasteur pipette of fixative while holding the slide at a 45° angle. Drain excess fixative from the slide by tapping the edges on a dry paper towel and place the slide at a 45° angle until dry. The fixative should evaporate from the slide within 60–90 s.
7. Once the slide has completely air-dried, remove it from the drying chamber and assess cell density and metaphase spreading using a phase contrast microscope (*see Notes 12–16*).

3.3 Chromosome Staining

1. Place the slides on a slide warmer at 90 °C for 60 min (*see Note 17*).
2. Arrange five Coplin jars containing their respective solutions in the following order: 1× trypsin banding solution (50 mL), tap water (~45 mL), working stain solution (44 mL), and the final two Coplin jars both containing reverse osmosis (RO) purified water (~45 mL) (*see Note 18*). Slides will be treated for banding and staining using these five Coplin jar solutions sequentially.
3. Begin slide banding and staining (*see Note 19*). Immerse aged slides in 1× trypsin banding solution for 30 s and then immediately immerse slides in tap water for approximately 3 s. Following tap water immersion, tap edges of slides on a paper towel to remove excess water. Immerse slides in working stain solution for 1 min and 30 s. Finally, immerse slides in both jars of filtered water sequentially for approximately 3 s each.

4. Tap edges of slides on a paper towel and blow compressed air over both sides of the slide to dry.
5. Check the appearance of the chromosomes in a brightfield microscope (*see Notes 20–23* and Fig. 2).
6. Add 2–3 drops of Cytoseal 60, and place coverslip over the slide. Allow the adhesive to dry for at least 16 h (overnight) before analysis.

3.4 Chromosome Analysis (See Note 24)

1. Review sample information prior to analysis; in particular, check the cell line sex (if known), reason for testing, and previous cytogenetic results (if applicable).
2. Ensure chromosome morphology and resolution are adequate for analysis (*see Note 25*).
3. Scan the slide using the 10 \times objective in a consistent, repeatable pattern, e.g., starting at the end closest to the label and scanning up and down while moving to the left in the field of view.
4. Select metaphases that appear to be readable, i.e., chromosomes can be counted and identified (*see Note 26*).
5. Image metaphases using the acquisition software and the camera mounted to the brightfield microscope, using either the 60 \times or the 100 \times objective.
6. Count a minimum of 20 cells, analyze a minimum of eight cells, and karyogram a minimum of four cells (*see Notes 27 and 28*). Document cell results using the International System for Human Cytogenomic Nomenclature (ISCN) [14] and record details of the analysis (*see Note 29*).
7. If a numerical recurrent acquired chromosome abnormality (*see Notes 30–32*) occurs once in the first 20 cells examined, expand analysis to establish clonality (*see Note 33*). Increase the number of cells to be counted from 20 up to 40 cells. Stop counting if the same abnormality is seen again.
8. If a structural recurrent acquired abnormality (*see Notes 30–32*) occurs once in the first 20 cells examined, expand analysis to establish clonality (*see Note 33*). Score up to an additional 20 cells (*see Note 34*). Stop scoring if the abnormality is seen again. Count any abnormal cell(s) found while scoring so that they can be included in the results.
9. Upon completion of the analysis, compare chromosomes from all karyogrammed cells using analysis software.
10. Record the summarized results of all counted cells by listing individual clones with the number of cells in brackets, per ISCN guidelines [14]. List nonclonal (single cell) findings separately from the main results.

3.5 Results

Interpretation (See Note 35)

1. Review all chromosomes in the karyogrammed cells.
2. Review the cells with clonal and nonclonal abnormalities.
3. Designate the final karyotype results as normal or abnormal (*see Notes 36 and 37*). Certain cytogenetic changes are considered by WiCell Characterization to be preparation artifact or polymorphic variants and are not typically designated karyotypically abnormal (*see Note 38*).
4. Record the overall interpretation of results (*see Notes 39 and 40*).

4 Notes

1. Upon receipt of the culture, create a unique identifier (e.g., a six-digit number) for tracking purposes and record the following information: investigator's name and contact information, date and time of receipt, cell line name, cell line passage number, cell line species, cell line sex, cell type (e.g., ESC or iPSC), culture vessels (e.g., 6-well plates or T25 flasks), culture matrix, culture medium, previous genetic test results (if any), and any additional information provided by the submitting investigator. Assess the cell culture using a phase contrast microscope and document culture status, including culture confluence and colony characteristics such as shape and size. Additionally, inspect the cell culture for any signs of microbial contamination.
2. Determine if the cell culture is ready for harvest (Fig. 1). If the cell culture does not meet harvest requirements, continue culturing until requirements are met or request a replacement sample. If the hPSC culture is shipped overnight from another location, allow at least 16–24 h of recovery time in an onsite culture incubator prior to harvesting. Filter supplied media prior to culture feeding/media replenishment. Culture sample in accordance with submitting investigator's instructions. As the harvest protocol arrests cells at metaphase for chromosome analysis, cells must be robustly dividing. Harvest should be avoided on cell cultures at lower confluence (<40%) as this yields an insufficient number of cells in metaphase. Overgrown cultures (>90% confluence) should also be avoided as it slows cell growth and contains fewer cells in metaphase [20].
3. One 6 mL aliquot of 1× trypsin-EDTA is sufficient for 6 wells (or two T25 flasks) of cells to be harvested. One 5 mL aliquot of trypsin stop solution is required for each sample to be harvested. An aliquot of 5 mL of hypotonic solution is required for each sample to be harvested.

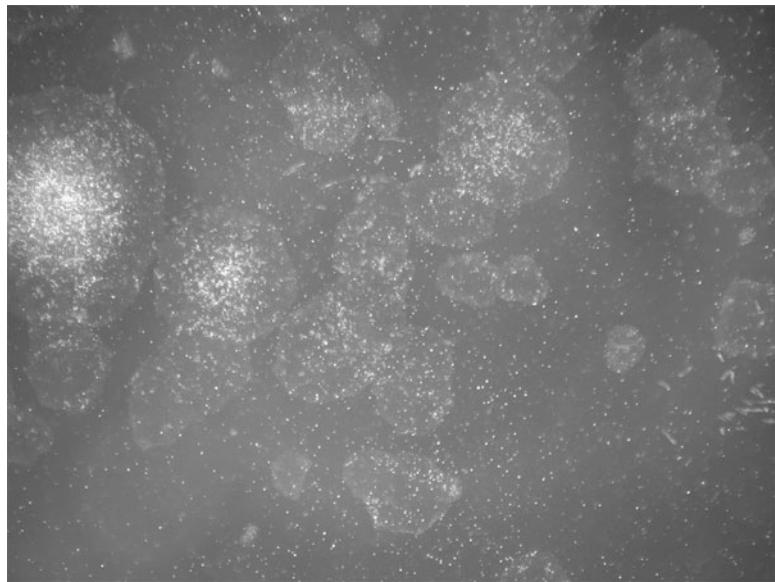


Fig. 1 Ideal confluence of cell culture for harvest. Optimal confluence ranges between 40% and 60%, and a minimum of 3–5 millions of cells are required, i.e., cells from 3 wells of a 6-well plate or equivalent (e.g., one T25 flask)

4. Colcemid is a mitotic inhibitor that arrests dividing cells during metaphase, thereby allowing for chromosome analysis. Ethidium bromide is a DNA intercalating agent used to counteract the chromatin condensation effects of colcemid and maintain optimal chromosome band level (resolution). Both reagents require accurate working concentrations (10 µg/mL for ethidium bromide and 200 ng/mL for colcemid) to achieve the desired effects.
5. Transfer the three pre-warmed reagents from the 37 °C water bath to the biosafety cabinet after cleaning them with 70% ethanol. Add 1 mL of pre-warmed 1× trypsin-EDTA working solution to each well of cells to be harvested for a 6-well plate, or 3 mL into one T-25 flask. Do not allow trypsin digestion to exceed 9 min.
6. Use an inverted phase contrast microscope, if needed, to confirm detachment of cells from the culture vessel surface or look for detachment through the top of the culture vessel. If gentle pipetting is not sufficient to detach the cells, use a glass serological pipette or a cell scraper to scrape off the cells. Pipette the cell suspension up and down within the centrifuge tube approximately five times to break up the cell clumps.
7. Hypotonic solution swells cells and spreads the chromosomes, thereby minimizing chromosome overlap and facilitating a more effective analysis. Manually agitating the centrifuge tube

while adding the hypotonic solution dropwise may prove challenging at first. An effective technique is to grip the top of the centrifuge tube between the index finger and thumb, then insert the serological pipette partway into the tube and rattle the rim of the tube around the pipette. Note that when using this technique, the serological pipette comes into contact with specimen cells and must be replaced immediately thereafter.

8. This step is referred to as “pre-fixation”; exposing cells to this small amount of fixative solution stops the cell swelling. Add the fixative from above the tube where it is less likely that your pipette tip will touch specimen cells; if the pipette tip accidentally touches the tube, replace it with a new tip.
9. Fixative preserves cells while removing proteins and debris. At this point, samples are said to be in “first fix.” If needed, they can be placed in a 4 °C refrigerator for several days (e.g., a harvest started during the weekend) before proceeding with subsequent fixative changes and slide preparation. Fixed cells can be stored at 4 °C for up to 2 weeks and long term at –20 °C.
10. The technologist empirically determines the optimal amount of fixative. The cell suspension should appear slightly cloudy. A general rule is 1 mL of fixative per well of cells harvested from a 6-well plate. If too much fixative is added, then centrifuge the tube for 5 min at $200 \times g$, and aspirate the supernatant; tap the tube to resuspend the cell pellet, and reattempt by adding fixative more slowly.
11. Chromosome spreading is a function of drying time and is influenced by humidity and temperature [21], which must be tightly regulated within a controlled chamber. Turn the cytogenetic drying chamber on at least 1 h prior to slide preparation.
12. Usually, three slides are prepared per case; the number of slides to be prepared depends on the size of the cell pellet, the mitotic index of the sample, and the extent of the analyses.
13. If the cell density is too high (more than approximately 100 nuclei per $10\times$ field of view on a phase contrast microscope), add several drops of fixative to the cell suspension in the 15 mL conical tube, and repeat the slide preparation steps on a new slide.
14. If the cell density is too low (less than ten nuclei per $10\times$ field of view), add additional drops of the cell suspension to the remaining slides. If necessary, centrifuge the 15 mL conical tube containing the cells at $200 \times g$ for 5 min; aspirate the excess fixative; resuspend the pellet in less fixative than added initially, and repeat steps with a new slide.

15. If there are under-spread metaphases (the majority of chromosomes are overlapped and indistinguishable), then increase humidity or decrease temperature inside the drying chamber to slow slide drying. Alternatively, adjust the ratio of methanol to acetic acid in the fixative to 2:1.
16. If there are overspread metaphases (cell boundaries are not distinguishable), then decrease humidity or increase temperature inside the drying chamber to accelerate slide drying. Alternatively, adjust the ratio of methanol to acetic acid in the fixative to 4:1.
17. Slides from differentiated cell types (e.g., fibroblasts) can be aged at 90 °C for 60 min, and then at 60 °C overnight to improve banding patterns; note that this increased aging may harden chromatin and necessitate longer trypsinization time during slide staining.
18. Trypsin enzymatically digests the chromosomes and slide immersion in tap water will halt this enzymatic action. Leishman solution stains GC-rich euchromatin lightly and AT-rich heterochromatin darkly [14] and purified water washes off excess stain. This process yields alternating dark and light bands that provide the basis for chromosome analysis.
19. First, stain one test slide to establish the optimal conditions for banding and staining.
20. If the chromosomes are under-trypsinized (chromosomes appear dark with indistinct bands, or cytoplasm is visible over metaphase cells) increase the amount of time that slides are immersed in 1× trypsin banding solution by 5 s and retest.
21. If the chromosomes are over-trypsinized (chromosomes appear swollen and “fuzzy”) decrease the amount of time that slides are immersed in 1× trypsin banding solution by 5 s and retest.
22. If the chromosomes are under-stained (chromosomes appear faint and light but have identifiable banding patterns) increase the amount of time that slides are immersed in stain working solution by 20 s and retest.
23. If the chromosomes are overstained (chromosomes appear overly dark but have identifiable banding patterns), decrease the amount of time that slides are immersed in stain working solution by 20 s and retest.
24. Chromosome analysis is performed by manually inspecting the unique banding pattern of each chromosome. It is technically challenging and typically requires training through a clinical education program. In clinical cytogenetic laboratories, technologists that perform chromosome analysis maintain professional credentials issued by a recognized body, such as the American Society for Clinical Pathology (ASCP).

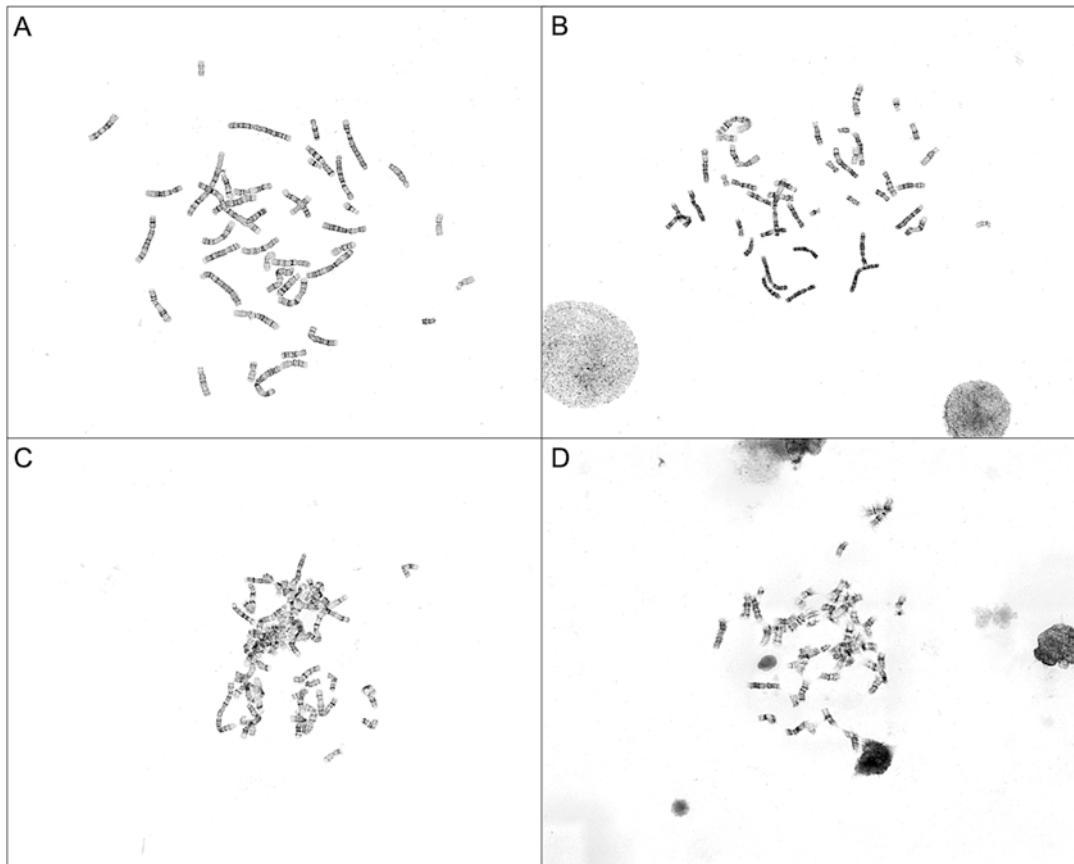


Fig. 2 Stained metaphase spreads of varying chromosome morphology and spreading. **(a)** A metaphase nucleus exhibiting ideal chromosome spreading and morphology. **(b)** A metaphase nucleus with differential staining; note the optimal staining in the upper region whereas chromosomes in the lower region are overstained and too dark. **(c)** A metaphase nucleus with optimal staining but under-spread chromosomes. The heavy overlapping obstructs chromosome banding patterns and therefore analysis. **(d)** A metaphase nucleus that has been over-trypsinized, note the “fuzzy” edges of the chromosomes

25. Prepare additional slides if chromosome morphology and/or length is inadequate or more slides are needed to obtain a complete analysis. Resolution should be appropriate to the indication for testing and the cell type: hPSC band resolution should be 450 or above to improve detection of subtle structural abnormalities. Duplications of chromosomes 1q32, 12p13.3, and 20q11.21, and deletions of chromosome 18q21 can be particularly subtle and therefore challenging to detect during analysis (*see Fig. 4*).
26. Do not select cells for analysis solely on the basis of good chromosome morphology. Omit cells from analysis only if extremely poor morphology or overspreading precludes chromosome identification. When cells are skipped because of poor morphology, attempt to identify structurally abnormal

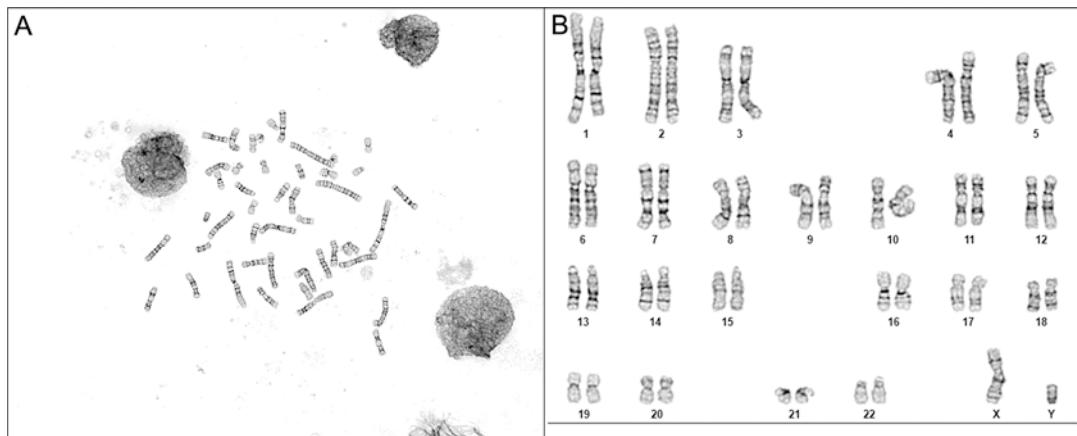


Fig. 3 A metaphase nucleus and corresponding karyogram. **(a)** A metaphase nucleus exhibiting ideal chromosome spreading and morphology. **(b)** The chromosomes from the same metaphase “cut out” and arranged into a karyogram

chromosomes, particularly if a specific recurring chromosome abnormality occurs in the cell line being analyzed.

27. All cells are counted to confirm a normal diploid number of 46 chromosomes, with two copies of each autosomal chromosome and two sex chromosomes (either XX or XY), to ensure there are no numerical abnormalities. Counting can also typically detect structural abnormalities unless they are especially small or subtle (e.g., duplication of 20q11.21, *see Fig. 4*). Eight of these counted cells are also analyzed by performing a band-to-band comparison of homologous chromosomes, which aids in the detection of subtle structural abnormalities. Of the eight analyzed cells, bands must be visible in each chromosome pair at least once. Finally, four of these analyzed cells are also karyogrammed wherein chromosomes are “cut out” from the metaphase spread and arranged next to their homolog using a specialized analysis software (*see Fig. 3*).
28. Karyogrammed cells should be representative of the overall case, and therefore priority for which cells to karyogram depends on the final test results. If summarized case results are normal, karyogram four normal cells. If case results are abnormal and all cells are abnormal: karyogram four cells, with at least one karyogram for each abnormal clone and priority given to the predominate abnormal clone. If overall case results are abnormal and there are normal and abnormal cells: karyogram three abnormal cells and at least one normal cell. If overall case results are abnormal and there are normal and multiple abnormal clones: karyogram four cells, with at least one cell from each abnormal clone (priority given to the

predominate abnormal clone) and at least one cell from the normal clone.

29. Record the following information for each cell: technologist name, date, slide number, cell coordinates, cell karyotype, comments specific to the cell, cell quality, and analysis state (i.e., counted, analyzed, karyotyped). Cell karyotype should be recorded in the following format: chromosome count, sex chromosomes, description of cytogenetic abnormality (if any). Examples of cell karyotypes are listed below, *see Note 32* and Fig. 4 for additional examples of cytogenetic abnormalities.
 - (a) 46,XY: a normal chromosome complement of 46 chromosomes, followed by the sex chromosomes indicating a male cell in this case.
 - (b) 46,XX: a normal chromosome complement of 46 chromosomes, followed by the sex chromosomes indicating a female cell in this case.

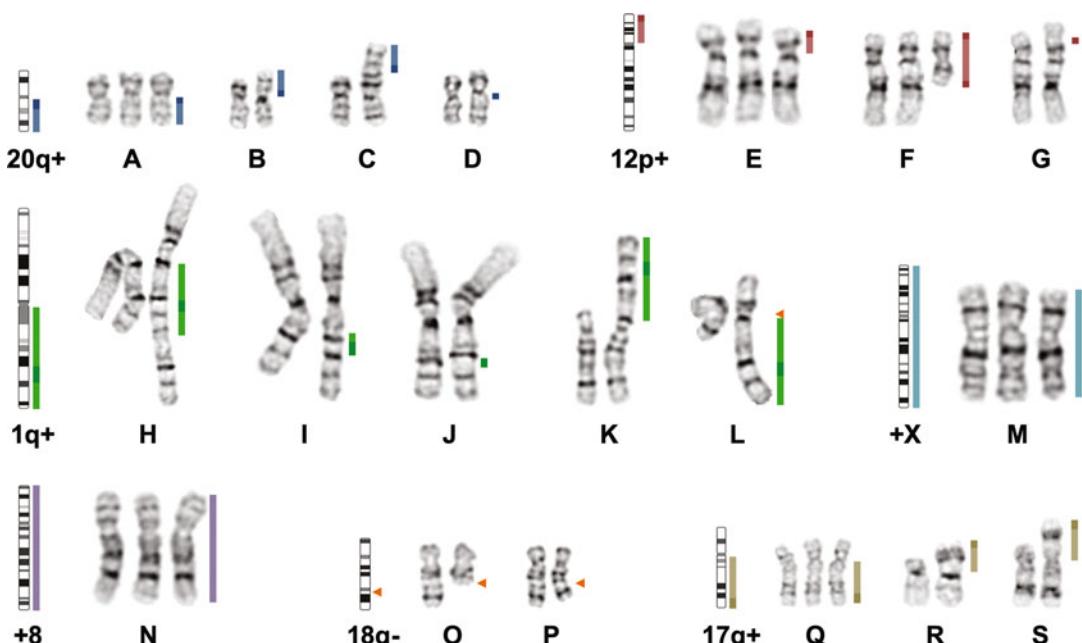


Fig. 4 Examples of recurrent acquired karyotypic abnormalities in hPSCs. Abnormalities can present with different sizes and mechanisms. Each aberration type is designated by a distinct color that outlines the chromosomal region gained or lost, and minimal overlapping regions (if known) are indicated by a darker color shade for cytobands 1q32, 12p13.3, 17q25, 18q21, and 20q11.21. Abnormalities are reported using ISCN: (a) +20, (b) i(20)(q10), (c) idic(20)(p12), (d) dup(20)(q11.2q11.2), (e) +12, (f) +i(12)(p10), (g) dup(12)(p13p13), (h) dup(1)(q21q44), (i) dup(1)(q25q32), (j) dup(1)(q32q32), (k) der(14)t(1;14)(q12;p11.2), (l) der(18)t(1;18)(q12;q21.1), (m) +X, (n) +8, (o) del(18)(q21.1), (p) del(18)(q21.1q21.3), (q) +17, (r) der(22)t(17;22)(q21.3;p11.2), (s) i(17)(q10)

- (c) 47,XY,+12: an abnormal chromosome complement due to an extra chromosome 12 (“+”); note the chromosome count of 47 in this case due to the trisomy 12 numerical abnormality.
 - (d) 46,XX,dup(1)(q25q42): an abnormal chromosome complement due a duplication (“dup”) of the long (q) arm of chromosome 1; specifically, gain of 1q segments from cytobands q25 to q42 (inclusive), which is a structural abnormality (note the normal chromosome number of 46 as this is not a numerical abnormality).
 - (e) 46,XY,der(22)t(17;22)(q21.3;p11.2): an abnormal chromosome complement due a derivative (“der”) chromosome 22 resulting from an unbalanced translocation; specifically, extra chromosome 17q segments (from q21.3 to qter) have translocated to the p-arm of chromosome 22 at cytoband p11.2.
 - (f) 46,XX,i(20)(q10): an abnormal chromosome complement due to an isochromosome (“i”) of the long arm of chromosome 20, resulting in loss of the short (p) arm and gain of the long (q) arm.
 - (g) 46,XY,del(18)(q21.1q21.3): an abnormal chromosome complement due to an interstitial deletion (“del”) of chromosome 18 long (q) arm segments from cytobands q21.1 to q21.3.
 - (h) 47,XX,+i(12)(p10): an abnormal chromosome complement due to an extra, structurally abnormal, chromosome; in addition to two normal chromosome 12s, there is a supernumerary isochromosome (“+i”) of the short arm of chromosome 12 resulting in four copies total of chromosome 12p.
30. Mark any abnormalities with an arrow (numerical or structural) in both the metaphase images and karyogram images.
 31. If an abnormality is seen at any point during analysis, check all other cells in the analysis, including all cells examined prior to detecting the abnormality. If the region or chromosome suspected to be abnormal is obscured in any previously examined cell, locate a different cell to analyze. This ensures that no abnormality will be missed in an examined cell because of preparation artifact such as chromosome overlap. Make a note that the metaphases were checked for that abnormality and do not have it.
 32. Based on internal data and published literature [2, 4, 7–9], the following cytogenetic changes are designated recurrently acquired in hPSC. Note that this is a working list based on the discretion of WiCell Characterization.

- (a) Gain of whole chromosome X.
 - (b) Gain of whole chromosome Y.
 - (c) Gain of chromosome 1, specifically 1q32, e.g., trisomy 1, duplication of 1q, +isochromosome 1q, or any derivative chromosome resulting in gain of 1q sequences.
 - (d) Gain of chromosome 7, specifically the p-arm, e.g., +isochromosome 7p.
 - (e) Gain of whole chromosome 8, e.g., trisomy 8.
 - (f) Gain of chromosome 12, specifically 12p13.3, e.g., trisomy 12, duplication of 12p, or +isochromosome 12p.
 - (g) Gain of whole chromosome 14, e.g., trisomy 14.
 - (h) Gain of chromosome 17, specifically 17q25, e.g., trisomy 17, duplication of 17q, or any derivative chromosome resulting in gain of 17q.
 - (i) Loss of chromosome 18, specifically any 18q21 deletion (del).
 - (j) Gain of chromosome 20, specifically 20q11.21, e.g., trisomy 20, duplication of 20q, isochromosome 20q, or any derivative chromosome resulting in gain of 20q.
33. Clonality is based on the number of cells that contain the same cytogenetic change. Two or more cells with the same extra chromosome is a clone. Three or more cells with the same missing chromosome is a clone. Two or more cells with the same structural abnormality is a clone. Conversely, non-clonality is one cell with an extra chromosome, one-to-two cells missing the same chromosome, or one cell with a structural abnormality.
34. Cell scoring is a limited evaluation for the presence or absence of a specific cytogenetic feature. As a limited evaluation, scored cells are not included in the summarized case results.
35. In a clinical setting, a laboratory director who performs cytogenetic results interpretation usually holds a doctoral degree, should have broad knowledge of human genetics, and is generally certified by a professional organization, such as the American Board of Medical Genetics and Genomics (ABMGG).
36. Designate the karyotype as normal or abnormal per ISCN guidelines [14] and indicate if abnormalities are recurrently acquired in hPSC (*see Note 32*). Final overall case results of 46,XX qualify as an apparently normal female karyotype and 46,XY qualify as an apparently normal male karyotype. Abnormal karyotype designations may still be within the acceptable range depending on context (e.g., a disease model line such as a

trisomy 21 induced pluripotent stem cell line generated from a Down syndrome patient).

37. If nonclonal (single cell) findings are present, indicate if the observed abnormalities are recurrently acquired in hPSC (*see Note 32*). Nonclonal findings may be due to technical artifact but can also be indicative of a low-level subpopulation; if this subpopulation contains a recurrently acquired abnormality, it may expand over time [4, 6].
38. In the experience of WiCell Characterization, 1–2 cells (per case) containing terminal deletions of chromosome 9q10-q13 are likely preparation artifact and are generally not considered abnormal. Furthermore, commonly observed polymorphic variants in hPSC include the classic pericentric inversions of chromosomes Y, 2, and 9. If present, such variants should be in all cells examined (non-mosaic) and typically do not prompt an abnormal karyotype designation.
39. Record the following information per case: name, date of interpretation, number of cells counted, analyzed, and karyogrammed, chromosome band level range (of karyogrammed cells), and karyotype results interpretation (normal or abnormal).
40. Examples of case karyotype results and corresponding interpretations are given below.
 - (a) 46,XX[20]: This is a normal karyotype; no clonal abnormalities were detected at the stated band level resolution.
 - (b) 46,XY,i(20)(q10)[2]/46,XY[18]: This is an abnormal karyotype. There is an isochromosome of the long (q) arm of chromosome 20 in two of twenty cells examined. This imbalance results in trisomy for 20q and monosomy for 20p. Gain of chromosome 20q is a recurrent acquired abnormality in human pluripotent stem cell cultures. No other clonal abnormalities were detected at the stated band level of resolution.
 - (c) 46,XX,dup(1)(q25q42)[18]/46,XX[2]: This is an abnormal karyotype. There is a duplication in the long (q) arm of chromosome 1 in eighteen of twenty cells examined. Gain of chromosome 1q is a recurrent acquired abnormality in human pluripotent stem cell cultures. No other clonal abnormalities were detected at the stated band level of resolution.
 - (d) 46,XY[39], Nonclonal findings: 47,XY,+12: This is a normal karyotype; no clonal abnormalities were detected at the stated band level resolution. There is a nonclonal finding, listed above, which contains a chromosomal

aberration (trisomy 12) recurrently acquired in human pluripotent stem cell cultures. An additional 20 cells were examined for this chromosomal aberration; it was not observed. Nonclonal findings may result from technical artifact, but may be due to a developing clonal abnormality or to low-level mosaicism.

Competing Interests

All authors were employees of WiCell during the drafting of this chapter.

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Chapter 17

Become Competent in Generating RNA-Seq Heat Maps in One Day for Novices Without Prior R Experience

Kejin Hu

Abstract

Heat map visualization of RNA-seq data is a commonplace task. However, most laboratories rely on bioinformaticians who are not always available. Biological scientists are afraid to prepare heat maps independently because R is a programming platform. Here, using RNA-seq data for 16 differentially expressed genes in *WNT* pathway between embryonic stem cells and fibroblasts, I share a tutorial for novices without any prior R experience to master the skills, in one day, required for preparation of heat maps using the *pheatmap* package. Procedures described include installation of R, RStudio, and the *pheatmap* package, as well as hands-on practices for some basic R commands, conversion of RNA-seq data frame to a numeric matrix suitable for generation of heat maps, and defining arguments for the *pheatmap* function to make a desired heat map. More than 20 template scripts are provided to generate heat maps and to control the dimensions and appearances of the heat maps.

Key words Heat maps, RNA-seq, RStudio, *pheatmap*, Tutorial, WNT pathways, Human embryonic stem cells

1 Introduction

RNA sequencing (RNA-seq) has become a commonplace technology for the biological and biomedical research laboratories [1–4]. RNA-seq generates genome wide transcription data. It is challenging to present the differential expressions for such a large set of genes. One popular method for visualization of RNA-seq data is heat map presentation using a color gradient. However, preparation of high-quality RNA-seq heat maps usually requires a programming platform. The widely used programming platform for generation of heat maps of RNA-seq data is R. Most biological and biomedical scientists are afraid to try the programming platforms for generation of RNA-seq heat maps, and rely on

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bioinformaticians who may not understand the biological problems well, and are not always conveniently available.

Here, I share a laboratory protocol designed for biological scientists without any prior experience in R to quickly become proficient in preparation of heat maps using the R platform. In a single day, audiences will be able to prepare professional heat maps to visualize RNA-seq data. There are several available packages for preparing heat maps [5–8]. This protocol uses the pretty heat map package *pheatmap* [6]. In the morning, the audiences will be guided to download and install R and RStudio onto their computers, and then practice some essential R commands, functions, and scripts prior to generating heat maps. In the afternoon, audiences will generate heat maps using a demo RNA-seq data frame. More than 20 template scripts along with heat map figures with different features are provided for novice users even without prior experience of R to generate heat maps and to manipulate the shape and appearance of the heat maps. This tutorial aims to turn a difficult and lengthy learning process into an easy, short, and enjoying experience. The scripts provided in the tutorial also serve as a handy reference for the more experienced users. It is a great refresher for the infrequent users of R and the heat map package.

2 Materials and Equipment

2.1 Computers (PC Windows or Mac OS X) and Softwares

The RStudio 1.2 requires a 64-bit PC operating system and works exclusively with the 64-bit version of R. This protocol was written based on the latest version of RStudio 1.2.1335 associated with the R 3.6.1 on platform of Microsoft Windows 10 Enterprise, and runs well on Windows 7 (64-bit), as well as with the older version RStudio 1.1.463 associated with the R3.3.3 on iMac OS X 10.9.5. The R and the *pheatmap* package were downloaded from The Comprehensive R Archive Network (CRAN) repository. A free version of RStudio was downloaded from www.rstudio.com.

2.2 The DESeq2 Read Count Data Frame

The demo data are based on real RNA-seq experiments in the author's laboratory, and some have been published [4]. The RNA-seq experiments sequenced four human fibroblast samples, and three human embryonic stem cell (ESC) samples. The format of the table is similar to what a bioinformatician generates using DESeq2 and passes to his/her biological scientists. For the convenience of presentation, the table includes only 16 genes in the WNT signaling pathways so that the heat maps in the tutorial appear larger, clear, and legible. Ten of the genes have at least two times higher expression in ESCs, while the remaining six have at least two times higher expression in human fibroblasts. All of the 16 genes have q values less than 0.01. The normalized read counts cover a large range from 1.7 to 7,659, and the log₂ fold changes

range from -6.9 to 11.6. Many genes are expressed only in one cell type. A gene is empirically deemed not expressed in a cell when the average normalized read counts are less than 50 [2, 4].

The original table is available as supplementary material. It is also available from the author upon request. If it is difficult to download the original RNA-seq data RNAseqHeatmap_wnt.csv, you can make a short table in Excel using the data in Table 1 and save the data in csv format.

After becoming proficient by practicing this protocol, users can generate heat maps with their own data with similar RNA-seq data frame in the format of .csv. One should convert his/her RNA-seq read count table in the Excel file to a comma separated values (csv) format. This can be done easily in Excel using the “Save As” function.

2.3 Download R and RStudio

RStudio is a user-friendly version of R. We will use RStudio to generate heat maps because it is more convenient to use. RStudio requires R to function. Therefore, we have to download R first. All commands and functions are the same in R and RStudio and you can practice in either one or in both after you will download the softwares onto your computer. The processes for downloading R and R Studio are provided in the following sections.

2.4 Downloading R

The steps may vary depending on the types of your computer systems, PC Windows, or Mac OS X. You may have to ask the IT personnel to help if unexpected problems occur.

1. Go to www.r-project.org/.
2. Click “CRAN mirror” under “Getting Started” subtitle.
3. Find the nearest mirror to you. In my case, it is National Institute for Computational Sciences, Oak Ridge, TN. Click on the link: <https://cran.nics.utk.edu/cran/>.
4. In the next page, click Download R for Windows.
5. In the R for Windows page, click “Install R for the First Time”.
6. Click on “Download 3.6.1 for Windows”. This is the latest version of R on the time of this protocol written, released on 2019-07-05. The version may be newer at the time of your downloading.
7. You will see a dialog box. Click Save.
8. In the dialog box, you will see a message of “R-3.6.1-win.exe” finished downloading. Click Open Folder.
9. In a new window, find the file of R-3.6.1-win and double click it.
10. You will see a new Windows dialog box asking “Do you want to allow this app to make changes to your device?”. Click Yes.

Table 1
Normalized read counts of RNA-seq data

external_gene.x	Fibroblast_1	Fibroblast_2	Fibroblast_3	Fibroblast_4	ESC1	ESC2	ESC3
MYCN	5.021377	1.703858	0	0	6104.348	3031.534	4661.45
PCDH1	5.503161	8.065378	13.17402	26.95382	6143.231	3574.371	7659.542
CTNNA2	2.020892	8.358199	5.957563	0	456.1059	410.3579	170.5276
PCDH11	18.78246	0	11.41216	16.11147	362.9158	184.3101	717.943
PCDHB16	30.09837	17.23551	35.65102	60.80093	339.4942	401.5489	429.4128
AXIN2	95.74123	122.3081	103.293	240.7407	1662.648	469.3609	1558.177
FZD9	18.21673	32.58625	12.29648	29.80337	118.6903	179.4875	49.63852
FRZB	241.2931	155.9666	61.00167	155.9059	1122.096	289.2833	190.3868
ADSS	2152.163	2494.122	2069.988	1857.153	4914.726	4859.624	4350.743
BMPRIA	1771.996	1030.246	2136.186	2000.28	3813.509	3350.53	3679.227
PPARD	2968.091	3330.526	3352.589	3989.729	929.0336	936.9425	922.8081
PCDHGAI0	1104.69	645.9197	855.0272	1412.63	103.5171	384.8895	126.412
KREMEN1	1589.04	2297.112	584.7121	1178.002	176.29	211.9809	117.5155
PCDHG5	123.0361	83.06663	180.6374	172.8532	6.188486	13.55698	17.84386
CDH13	3797.71	4029.024	1761.97	2025.046	53.61891	79.34874	40.25633
WNT5B	5736.458	5881.559	4439.834	4165.487	39.47173	46.66308	37.72429

11. In Select Setup Language window, select English, and Click OK.
12. Click Next in the Information window.
13. Click Next to install the R in the folder, C:\Program Files\R\R-3.6.1.
14. Select the component you want to install in the Select Components window. Deselect 32-bit Files and keep the others because we will use RStudio to generate heat map and RStudio 1.2 requires R and works exclusively with the 64-bit version of R. You can install all components though.
15. In the Startup Options window, click Next using the defaults option.
16. Click Next with Start Menu folder as R.
17. In the Select Additional Tasks window, check Create a desktop shortcut, and click Next.
18. Click Finish in the next window.
19. Now, you will see an icon for R on your computer desktop.
20. Double click the R icon to open it and you can start to practice as instructed below.

2.5 Downloading RStudio

RStudio requires R to function. You have to download and install R before you can work on RStudio. However, with R installed on the same computer you do not need to open R to use RStudio.

1. Go to www.rstudio.com.
2. Click the “Download RStudio” button.
3. Choose the free download version and Click DOWNLOAD.
4. In Installers, click “RStudio 1.2.1335—Windows 7+(64-bit)”, or the latest version at the time of your downloading.
5. Click Save in the pop-out dialog window.
6. Click “Open folder” in the dialog window.
7. In the new window find out the downloaded file, RStudio—1.2.1335, double click this file name.
8. Click Yes in the new dialog window to install.
9. Click Next in the RStudio Setup window.
10. Click Next in Choose Install Location window.
11. Click Install in the Choose Start Menu Folder Windows.
12. Click Finish in the final window to complete the installation.
13. Now, open RStudio and practice the R exercises provided below.

2.6 Know R by Hands-on Practice

Before Generating Your Own Heat Maps

You may be able to make heat maps following instructions in the next subheading, i.e., Sect. 2.7 without practicing R commands provided in this section. However, it is a good idea to familiarize yourself with some basic commands, R operators, concepts, and functions before you generate heat maps if you do not have any prior experience with R. Readers can gain more R experience by practicing my recent R tutorial [10].

R is a programming platform and uses commands. When you open R, there is a prompt sign > in the console window. This is where you type your commands to conduct data calculation and manipulation. We will briefly practice some basic R commands and functions so that we can better understand the scripts used in generating RNA-seq heat maps. All executing R commands and scripts will be indicated by blue text. Notes are given after a pound sign # following the corresponding R commands or scripts.

I encourage the audiences to read this tutorial in front of your computer and do what the tutorial instructs you to. Please note that you have to hit the Enter key to execute an R function or script, which will not be indicated in the following text. For clarity, the output of your executions will not be included in the protocol and you will see those in your R pane. R uses a lot of functions to execute various tasks. An R function is a special R object, which consists of a set of codes to execute a specific task/calculation. Let us start with the *date()* function on the first day of your R and *pheatmap* experience. Type *date()* after the R prompt sign >, which will be omitted for all the commands and scripts in this tutorial in order to save space, to find out today's date and the current time.

[date\(\)](#)

An R function includes its arguments in parentheses. Parentheses are needed even though no arguments are included (or default arguments). Now, type:

[date](#) # When parentheses of a function is missing only the contents of the function are printed.

Type *version* to see which version of R you are using:

[version](#) # the *version* function is an exception that it does not need parentheses.

To see what is the working directory the R is using, type:

[getwd\(\)](#)

One very useful command is the help function: *help()*. R has an inbuilt help facility we can use to find information and usage of an R function. For example, if you want to know the function of *date* you type the following after the prompt sign >:

[help\(date\)](#)

A new window will pop out. You will see detailed information about the function in question. The help page will describe the function in several sections which may include Description, Usage, Arguments, Details, Reference, See Also, Examples, Value, and Note. In RStudio (see below), this information will appear in the Files/Plots/Packages/Help/Viewer window. You can use the *help()* function for the commands used in this tutorial. For some R commands, you may have to enclose the command with quotation marks in the *help()* function:

```
help("+")      # An R help document for arithmetic operators will appear in the
                Help window. It returns a warning if + is not enclosed with
                quotation marks. Other arithmetic operators, the extract
                operator $, the assignment operators (=, <-, or ->), and the colon
                sequence operator: (see blow) also require quotation marks in the
                help() function.
```

R can execute mathematic calculations using the intuitive commands: +, -, *, /, and ^ for raising to a power (arithmetic operators); log, log10, log2, and exp (functions for logarithms and exponentials); sin, cos, and tan (trigonometric functions); sqrt, abs (miscellaneous mathematical functions), and others. Try the following calculations:

```
2 + 4
9 - 2
2*9
9/3
3^2
log2(2)
log2(0.5)
```

The results of the above operations (calculations) are printed out immediately on the screen of R console. Alternatively, you can store each result in an object. Try the following:

```
x = 2 + 4
y = 9 - 2
z = 2*9
h = 9/3
i = 3^2
g = log2(2)
f = log2(0.5)
```

The results of the above calculations are not printed out on the screen, but stored in the active memory as an R object with a given name for each calculation. Each of the above object names becomes

an R command now. You can type any of the object names to find out their contents. This is the simplest command in R.

```
x      # Typing an object name will return its contents.  
y  
z  
h  
i  
g  
f      # Typing an object name will return its contents.
```

The above commands of object names are an implicit use of the function `print()`. Try:

```
print(x)    # You will see the same result as you type x.  
print(y)    # You will see the same result as you type y.
```

An R object is temporarily stored in the active memory, and its content can be replaced by a new assignment. This operation affects the contents of the objects in the active memory, but not any object stored in your hard drive. Try:

```
x          # Its current content, 6, is printed.  
x = 4      # x is assigned a new value, 4.  
x          # The new content of x object, 4, is printed.  
x = 2*5 - 7 # x is assigned a new value again.  
x          # The current value of x, 3, is printed out.
```

Recall the previously used R commands using the up and down arrow keys on the keyboard. Please try to press the up arrow 7 times, and then the down arrow key to see what happens. It is useful because you may want to re-execute or modify the calculations you previously entered.

```
x = 2 + 3*4
```

Press the up arrow key and you will see “`x = 2 + 3*4`” appears on your screen. Now you can edit the existing one to get a different one, for example, change `x = 2 + 3*4` to `x = 2 + 3/3` by deleting `*4` and then adding `/3`. Then, hit the Enter key to see what will happen.

Instead of `=`, R commonly uses `<-` as an assignment operator. Try these:

```
x <- 10*2      # This is equivalent to x = 10*2.  
x  
y <- log2(2)    # This is equivalent to y = log2(2).  
y
```

R object names are case sensitive. Uppercase and lowercase of the same letter are different objects. Try:

```
A <- 4 + 6
a <- 4 + 2
A
a
```

Generate a numeric vector using the combine function *c()* and the colon sequence operator *:*. Type:

```
c(1:10)
```

An object can be a vector. You can generate the same vector above and store it in a vector object:

```
v <- c(1:10)      # This generates a numeric vector containing 1, 2, 3, 4, 5, 6, 7,
                    8, 9, 10, and store it in v.
v                  # print the vector of v on your screen.
```

We can log2-transform every element of the entire numeric vector.

```
log2(v)      # You will see the new vector containing the log2 values of the
                elements of the vector v.
```

We can repeat an element in a vector using the *rep()* function.

```
rep(2, 5)      # You will see that 2 is repeated by 5 times.
```

The above vector is numeric. We can generate a character vector using the combine function *c*.

```
c("female", "male")    # Character elements should be enclosed in quotation marks.
```

The above character vector can be stored in a vector object:

```
v1 <- c("female", "male")
v1
```

Elements of a character vector can be repeated as well using the *rep()* function.

```
v2 <- rep("male", 5)
```

We can use multiple functions to generate a vector, e.g., *c()* and *rep()* function.

```
v3 <- c(rep("female", 5), rep("male", 5))
v3
```

The major function of R is data analysis and manipulation. In R, there are many built-in data sets. You can find out what built-in data sets are included in the current version of R using the *data* function:

```
data()
```

An R-data set window will pop out containing a list of inbuilt data sets in R. This list of data sets will appear in the Source window if you practice in RStudio (see below).

You can print any available data set in R console by typing the data set name after the prompt sign. For example,

```
mtcars      # This matrix-name command is equivalent to the print(mtcars) command.
```

Use *summary()* and other functions to check the details of a data set.

<code>summary(mtcars)</code>	# The generic <i>summary</i> function returns a summary of the data set.
<code>head(mtcars)</code>	# The <i>head</i> () function returns the first several rows of the table so that a small table can give you an idea about the table. The numbers of rows can be defined. Try:
<code>head(mtcars, n = 5)</code>	# Print the first 5 rows of the data frame.
<code>head(mtcars, n = 3)</code>	# Print the first 3 rows of the data frame.
<code>min(mtcars)</code>	# The minimum function <i>min</i> () returns the minimum value in the data frame. This works only for numeric matrix or vector.
<code>max(mtcars)</code>	# The maximum function <i>max</i> () returns the maximum value in the data frame. This works only for numeric matrix or vector.
<code>ncol(mtcars)</code>	# The numbers-of-columns function <i>ncol</i> () returns the number of columns in the data frame studied.
<code>nrow(mtcars)</code>	# The numbers-of-rows function <i>nrow</i> () returns the number of rows in the data frame studied.
<code>dim(mtcars)</code>	# The <i>dim</i> ()function returns the dimension of the data frame, i.e. number of rows and columns.

We have seen numeric objects and vector objects above. An R object can be a matrix, a data frame, or a function. The mtcars is an inbuilt matrix object in R. You can take rows, or columns from a table/matrix using the index function `[]`, and assign the new matrix a different matrix object name.

<code>mtcars1 <- mtcars[, 1:4]</code>	# This script takes the first four columns of the mtcars matix and assigns the new matrix to a new matrix object name mtcars1.
<code>mtcars1</code>	# It prints the new matrix mtcars1 on screen.
<code>mtcars2 <- mtcars[1:5 ,]</code>	# This script takes rows 1 to 5 of mtcars and assigns the new data set a matrix object name of mtcars2.
<code>mtcars2</code>	# It prints out the matrix of mtcars2 on screen.
<code>mtcars3 <- log2(mtcars)</code>	# This script transforms the entire mtcars data set using log2, and assigns the log2-transformed data a new object name mtcars3. The data set to be transformed by log2 should be numeric.
<code>mtcars3</code>	# This command is equivalent to <code>print(mtcars3)</code> .

We can print specific column of the matrix using the extract operator \$.

```
mtcars$mpg # This script prints the mpg column of mtcars on screen. In Rstudio, when you type mtcars$, all column names will pop out for you to select.
```

We may want to sort a specific column. To sort, we use a combination of the *order* function, the index operator [], and the extract operator \$:

```
mtcars4 <- mtcars[order(mtcars$mpg),]
```

To examine the sorted matrix, print the new matrix:

```
mtcars4
```

The above script sorts the column of *mpg* from low to high. If you want to sort from high to low, the argument “decreasing = TRUE” should be included.

```
mtcars5 <- mtcars[order(mtcars$mpg, decreasing = TRUE),]
```

To check the sorted results, type:

```
mtcars5
```

Give new names to row or columns using the *rownames()*, *paste()*, and *sep* functions, and the colon sequence operator “:”:

```
rownames(mtcars) = paste("cars", 1:32, sep = "")
```

```
mtcars # It returns the table on your screen, and you will see that the row names have been changed to a new set of names ranging from cars1 to cars32.
```

You can remove the objects from the active memory using the function *rm()*.

```
rm(mtcars)
```

```
mtcars # You will see a table, but the row names become the original names because this is the inbuilt mtcars table, and you just removed the mtcars table you have assigned new row names.
```

```
mtcars1 # You will see the table of mtcars1 though because it is a different object although it is derived from mtcars.
```

```
rm(mtcars1)
```

```
mtcars1 # You will now see an error message: object 'mtcars1' not found. This is because mtcars1 was just removed from the active memory using the rm()function and mtcars1 is not an inbuilt data set.
```

To quit R, type:

```
q("yes") # R will ask if you want to "save workspace image". Click No. You can also quit your R session by clicking the "Quit session" in the pulldown menu under the "File" menu. A "Quit R Session" window pops out. Click "Save" or "Don't save", and R is quitted.
```

2.7 Generate Heat Maps in RStudio

The *pheatmap* package is an external one, which is not a part of the standard R installation, and it has to be installed before we can use it to generate heat maps. We use the *install.packages()* function to install it. Type:

```
install.packages("pheatmap") # After installation, please check the libraries window to make sure pheatmap is in the list.
```

The *pheatmap* package is widely used to prepare heat maps for RNA-seq data. *pheatmap* is simple, yet it has a lot of arguments, which allow users to control the graphical parameters. Readers are encouraged to have a copy of the package documentation [6] in their hands when practicing this section. The document gives description about each argument of the *pheatmap ()* function.

To find out which working directory RStudio is currently directed to, type:

```
getwd()
```

You may see a return like this: [1] “C:/Users/yourname/Documents”.

Now, direct the working directory to your heat map folder:

```
setwd("C:/Users/yourname/my1stpHeatmap")
```

You have to make the above folder on your computer beforehand. Please do not forget the “C:/”. If this is missing, you will have a message of “Error in setwd(“Users/yourname/my1stpHeatmap”)”. For folder name, it is not case sensitive. You can direct R to the same working directory by specifying *setwd(“c:/users/yourname/my1stpheatmap”)*. Please note the use of the lowercase letters of “c”, “u”, and ‘h’ here. These make no difference because they are just parts of folder names.

To confirm the change of working directory, type

```
getwd() # You will see, “C:/Users/yourname/my1stpHeatmap” on the screen.
```

Now, download the RNA-seq read-count file *RNAseqHeatmap_wnt.csv* from the MiMB website into your folder, *my1stpHeatmap*. This table is a data frame in R terminology and contains both numeric and character elements. The hyperlink (https://doi.org/10.1007/978-1-0716-1084-8_17) for downloading the demo RNA-seq data table: The demo table is also available from the author upon request. If it is difficult to download the original RNA-seq data *RNAseqHeatmap_wnt.csv*, you can make a short table in Excel using the data in Table 1, and save the data in csv format. In this case, you can skip the steps below, i.e., steps for extracting the desired columns, or dropping the unwanted columns.

To read your RNA-seq read-count file from the computer folder into R, use the *read.csv()* function:

```
read.csv("RNAseqHeatmap_wnt.csv")
```

The above function reads the file and print the table in RStudio. To save the table as a data frame in R (i.e., the computer active memory), we assign it a data frame object name, my1stHeatmap.

```
my1stHeatmap <- read.csv("RNAseqHeatmap_wnt.csv")
```

You will see your data frame name in the Environment window. Click on the data frame name my1stHeatmap on the Environment window, and your data will appear in the Source window. Please note that the extension of .csv is required in the file name RNA-seqHeatmap_wnt.csv for *read.csv* to function. If it is missing, you will see an error-warning message after running the *read.csv* function. When you will use your own RNA-seq data to generate heat maps, it is important to save your .xlsx file as a *.csv* file.

From the source window, you see the rows of the uploaded table use numbers as row names. We will make the gene names as row names so that the row names of your heat maps will be the informative gene symbols. To set the gene symbols as their corresponding row names, we use the *rownames()* function and the extract operator \$:

```
rownames(my1stHeatmap) <- my1stHeatmap$external_gene.x
```

Now, you see that the row names have been replaced with the corresponding gene names but the column of gene names, i.e., “external_gene.x”, still remain in the table. Please note that the new name of the data frame *my1stHeatmap* includes intentionally an uppercase H to demonstrate that R object name is case sensitive. If you use *my1stheatmap* here in which a lower case h is used, you will find an error warning, “*Error in rownames(my1stheatmap) <- my1stheatmap\$external_gene.x: object ‘my1stheatmap’ not found*”. Please note that your column header for the gene identities may be different from the one used in this template script. If this is the case, please use your column header for the gene symbol column after the extract operator \$ rather than external_gene.x used here.

The table (data frame in R terminology) contains many unwanted columns including the megadata. The following script will make a new matrix by taking the columns needed only for generation of heat maps, using the index operator [].

```
my1stHeatmap1 <- my1stHeatmap[, 10:16] # This script takes columns 10 to  
16 from the data frame  
my1stHeatmap to make a matrix  
table of 7 columns. The comma  
here indicates that all rows will  
be retained, and the colon sequence  
operator means from column 10 to  
16 will be extracted.
```

Alternatively, to make the working table for generation of heat maps, we can drop the unwanted columns from the original table

using the following script. This script uses the combine function `c()`, the index operator `[]`, and the colon sequence operator `:`.

```
my1stHeatmap1 <- my1stHeatmap[,-c(1:9, 17, 18)] # This script drops columns  
1 to 9, as well as columns  
17 and 18, leaving columns  
10 to 16 to make a new  
table of my1stHeatmap1.
```

If you have difficulty in downloading the original RNA-Seq table RNaseqHeatmap_wnt.csv, you can make a csv table by yourself using data in Table 1 in this chapter. Using this short table, you can skip the steps above for extracting columns 10 to 16 because the short table contains the required columns only.

Alternatively, you can delete the unwanted columns in Excel before you transfer the table from Excel to R.

Click on the new data set name `my1stHeatmap1` in the Environment window and display the new data in the Source window. Please note that the new table includes only 7 columns containing the normalized read counts for each repeats of the two cell types. Also, we keep the original table by assigning a new name for the new data set. The comma “,” inside the index operator `[,]` indicates that all rows will be retained in the new table.

Load `pheatmap` library. This can be done simply by clicking the box next to the `pheatmap` package in the Packages window, or by executing the `library()` function.

```
library(pheatmap) # You will see, after running this function, that the box  
next to the package name pheatmap become checked.
```

Generating the first heat map using the default arguments of the `pheatmap()` function.

```
pheatmap(my1stHeatmap1)
```

You will see your very first heat map in the Plots window (Fig. 1). However, the differentiation power is very low. The colors have to cover read counts from 0 to 7,659. For many genes, the differences between the embryonic stem cells (ESCs) and fibroblasts are not displayed well, for example, *FZD9*, *PCDHGC5*, *PCDHA11*, and *CTNN2*. But, we know there are at least two fold of differences for all the 16 genes between the two types of cells.

To find out the range of the read counts in the entire table, we use the `summary()` function.

```
summary(my1stHeatmap1)
```

You will see the data profile for each column on the Console window after running the above function. They are from 0 to 7,659 for the entire data set.

To solve this problem, we can transform the data using the `log2` function. Make sure you assign a new name for your `log2`-transformed data set.

```
heatmap_log2 <- log2(my1stHeatmap1)
```

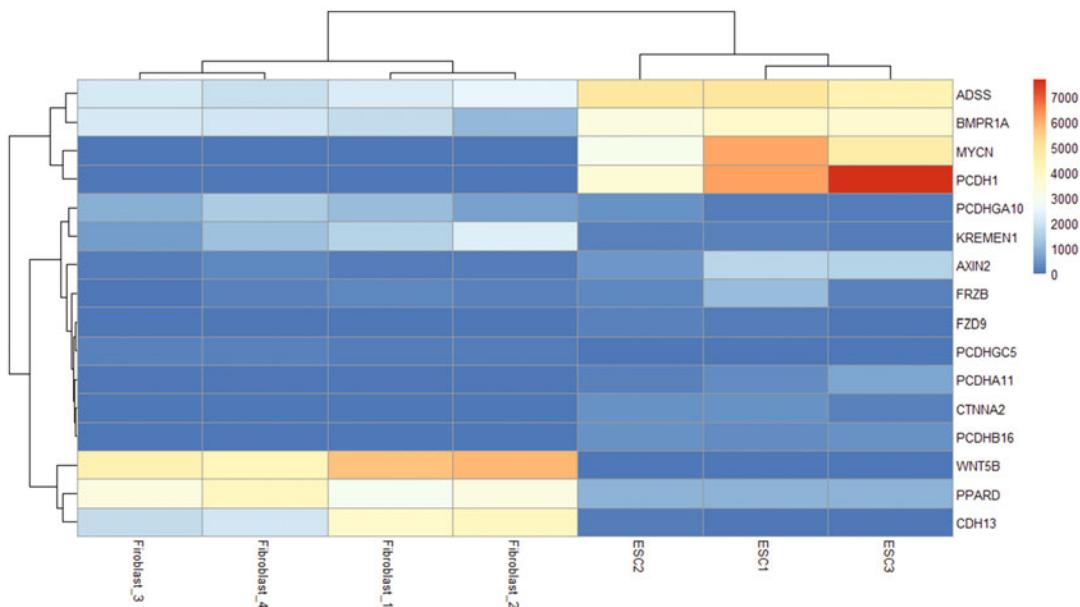


Fig. 1 A heat map prepared using the *pheatmap()* default arguments from the original normalized read counts without transformation and scaling

Run *pheatmap* again for the log2-transformed data.

```
pheatmap(heatmap_log2)
```

You will find an error message: “*Error in hclust(d, method = method): NA/NaN/Inf in foreign function call (arg 10)*”. This is because log2 transformation turns the zeros to “-Inf”. This can be seen using the *summary()* function.

```
summary(heatmap_log2)
```

To find the lowest log2 read count, we can sort each column using the *order()* function in combination with the index operator [], and the extract operator \$.

```
heatmap_log2[order(heatmap_log2$Fibroblast_1),]  
heatmap_log2[order(heatmap_log2$Fibroblast_2),]  
heatmap_log2[order(heatmap_log2$Fibroblast_3),]  
heatmap_log2[order(heatmap_log2$Fibroblast_4),]
```

Based on those sorting (Scripts for sorting for the three ESC columns are not given above), we found that the smallest transformed read count is 0.7688. Therefore, for practical purpose we replace each “-Inf” with a numeric element “-1” using index operator [] and the relational operator ==.

```
heatmap_log2[heatmap_log2 == -Inf] <- -1
```

Examine the table by scrolling around the table and using the *summary()* function.

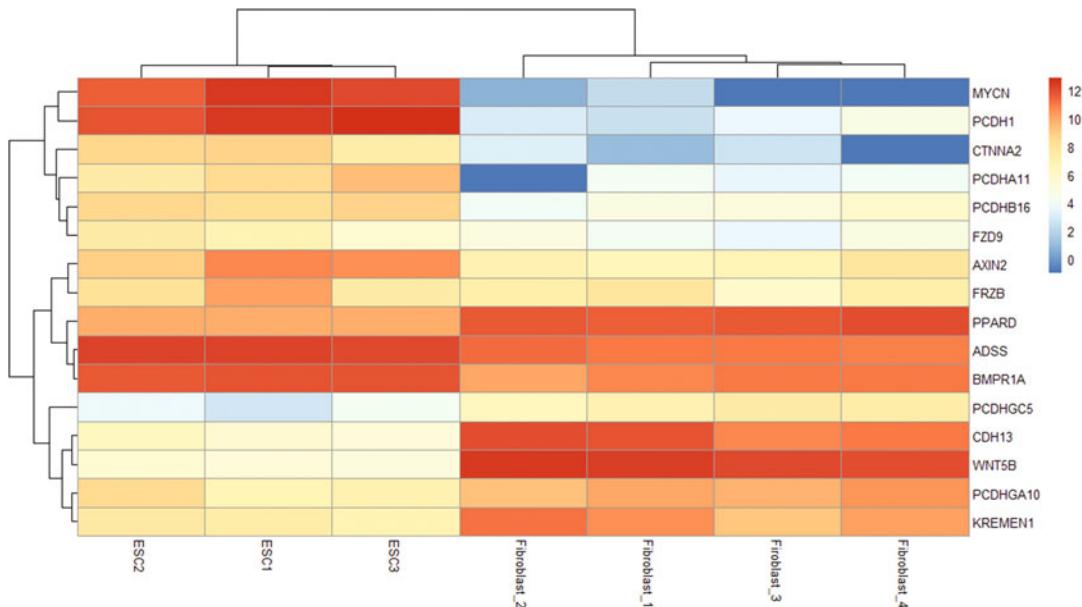


Fig. 2 A heat map prepared with the log2-transformed normalized read counts

Run the *pheatmap()* function again for the log2-transformed data.

```
pheatmap(heatmap_log2)
```

Now, you see a heat map with improved resolution using the same default color scheme (Fig. 2). For examples, the genes *FZD9*, *PCDHGC5*, *PCDHA11*, and *CTNN2* are now resolved well between fibroblasts and ESCs. The scale of the color legend is now ranging from 0 to 12. But, some genes are still not well resolved between these two types of cells, for example, *ADSS* and *BMPR1A*.

To further improve resolution, we can use the *scale* argument of the *pheatmap* function as follows:

```
pheatmap(heatmap_log2, scale = "row" )
```

Now, as you can see that every gene is resolved well between the two types of cells (Fig. 3). Please note that we use a comma to separate multiple arguments in an R function.

To save the heat map image on computer, you can just simply use the Export menu in the Plots window by clicking the pull-down menu and selecting “Save as Image”. A new dialog window will pop out. To finish the saving process, you can do these steps: Select the format of image file (png, jpeg, tiff, bmp, svg, or eps), give a file name, and change the size of image if necessary, then click the “Save” button. You can save your heat maps within any folder by choosing folder from the Directory button.

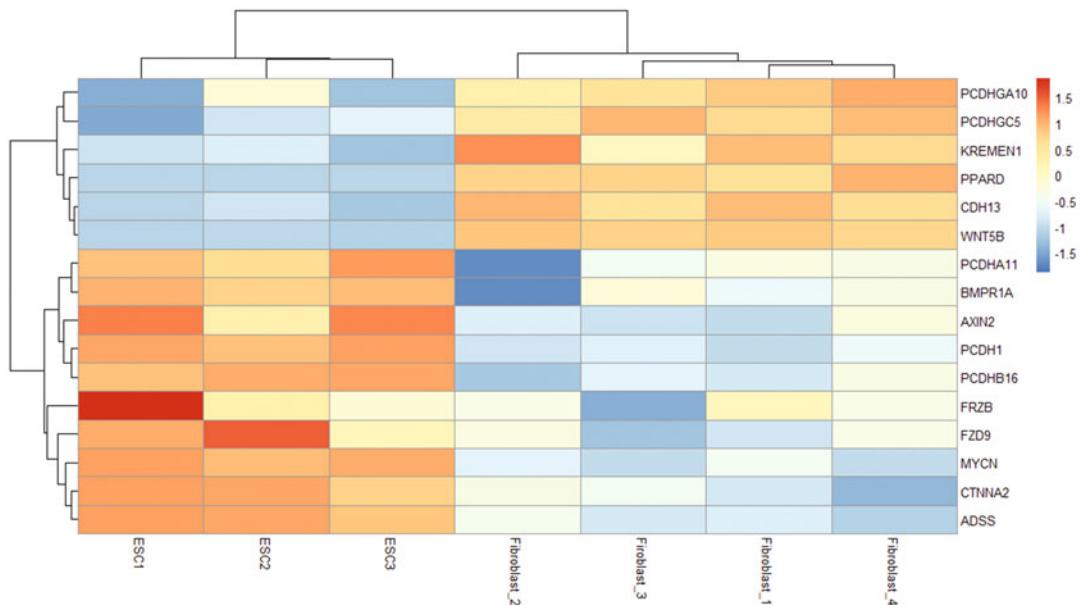


Fig. 3 A heat map generated with log2-transformed read counts and scaling of rows

Another way to save the heat map image is to include the *filename* argument:

```
heatmap(heatmap_log2, scale = "row", filename = "myRNAseqheatmap.pdf" )
```

This will save your heat map image in the current folder of the working directory. The format of the image is defined by the extension of a file name, and it can be png, pdf, tiff, bmp, or jpeg formats.

Or, you can save the heat map by using an R graphics devices *jpeg()*, *bmp()*, *png()*, or *tiff()* at three steps:

```
jpeg(file = "myjpegheatmap.jpeg")      # Open an R graphics device.  
heatmap(heatmap_log2, scale = "row")    # Create a heat map using the  
                                         # pheatmap function.  
dev.off()                                # Close the graphics device and return  
                                         # graphics device to the computer screen,  
                                         # i.e., the Plots window. This is  
                                         # important. Without running the  
                                         # dev.off() function, your image may not  
                                         # be saved correctly.
```

The heat map is now saved in the working directory. Go to the working directory to find out your newly generated heat map file. Other graphics devices work the same way as *jpeg()*.

After you have used a graphics device to save your image on computer, you may not see your image to show up in the Plots window when you run *pheatmap()* again for a new heat map. If this happens, you can solve this problem by calling *dev.off()* one more

time. You will see “null device”, which indicates all other devices are closed and the Plots pane becomes the active graphics device.

2.8 Change Dimensions and Appearance of Heat Maps

The heat map looks great now. For the purpose of presentation or publication, we may need to edit the heat maps to show or not to show the associated information in a desired way. We achieve this by defining additional arguments. In R, the arguments in a specific function can be in any order. In the following scripts, the arguments in discussion will be the last argument(s) in the *pheatmap()* function and is highlighted in bold face.

The shapes of dendrograms are affected by the methods of clustering and methods for calculating clustering distance. The default method for clustering in *pheatmap()* is complete linkage with the value of “*complete*”. Other available methods for clustering with *pheatmap()* include “*ward.D*”, “*ward.D2*”, “*single*”, “*complete*”, “*average*”, “*mcquitty*”, “*median*”, and “*centroid*”. Methods for clustering are based on the calculation of distance. The default distance measure in *pheatmap()* is “*euclidean*”. The *pheatmap()* also supports other distance measure methods for calculating dissimilarity/distance including “*correlation*”, “*maximum*”, “*manhattan*”, “*canberra*”, “*binary*”, or “*minkowski*”. The distance measure “*correlation*” is widely used in gene expression clustering considering that it focuses on expression behavior (upregulation or downregulation) rather than the levels of expression (Fig. 4). The default Euclidean method tends to cluster the genes with high values together and the genes with low values in separate groups.

```
pheatmap(heatmap_log2, scale = "row", clustering_method = "mcquitty",
clustering_distance_rows = "correlation") # This script gives a heat map with
                                         different shape of dendrogram and
                                         different arrangement of cells
                                         (Fig. 4).
```

We can show the expression levels for each gene in number using the logical argument of *display_numbers* (Fig. 5).

```
pheatmap(heatmap_log2, scale = "row", display_numbers = TRUE)
```

The size of the heat map may not be optimal. We can include the *cellwidth* argument to adjust the width of the cell to obtain a desired size of the heat map (Fig. 6):

```
pheatmap(heatmap_log2, scale = "row", display_numbers = TRUE, cellwidth = 22)
```

Now, the cell width is just enough to accommodate the numbers inside, but the cell height may not be optimal. We use *cellheight* argument to make them just fit (Fig. 7).

```
pheatmap(heatmap_log2, scale = "row", cellwidth = 22, display_numbers =
TRUE, cellheight = 20)
```

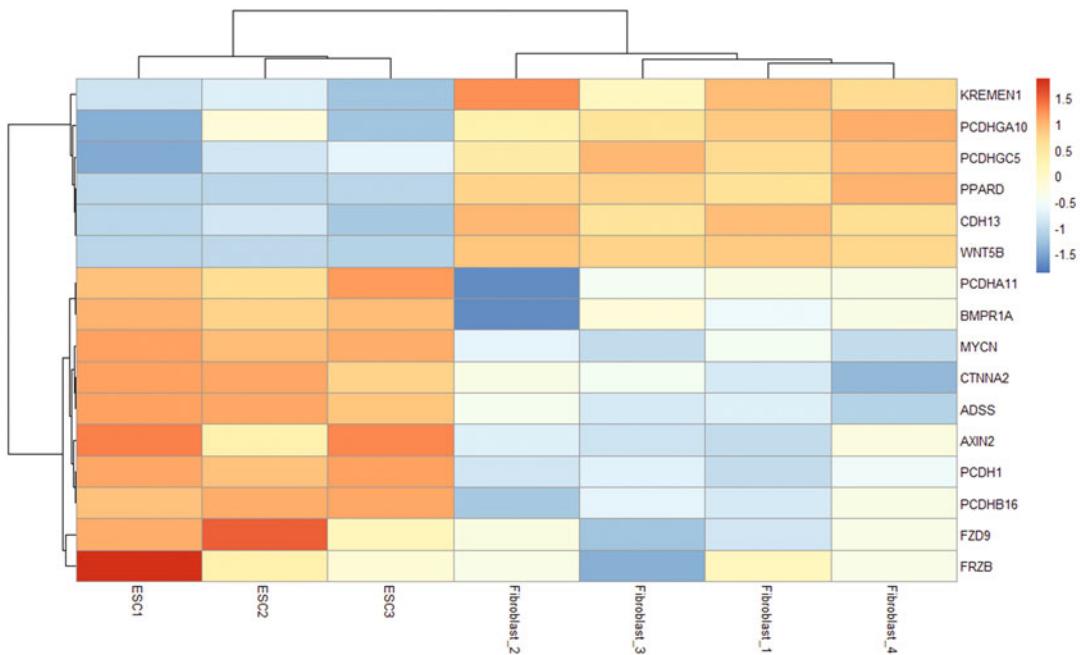


Fig. 4 A heat map with a different clustering method (“*mcquitty*”) and distance measure (“*correlation*”) from the default setting (“*complete*” and “*Euclidean*”, respectively)

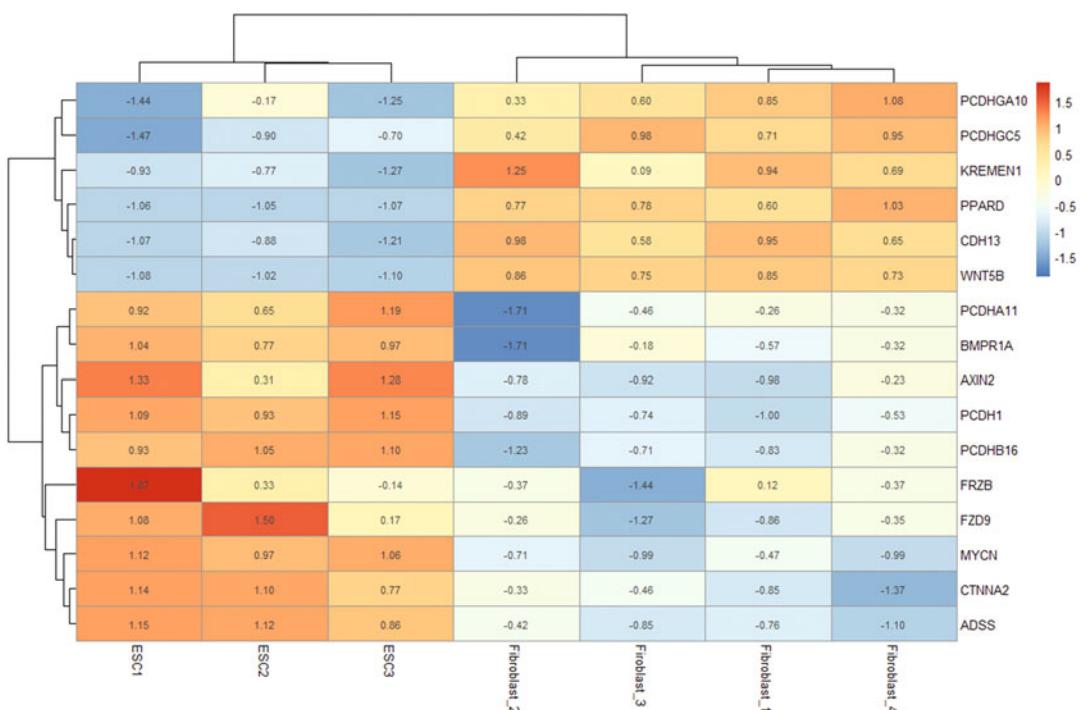


Fig. 5 The same heat map as in Fig. 4, but with the expression levels shown in each cell

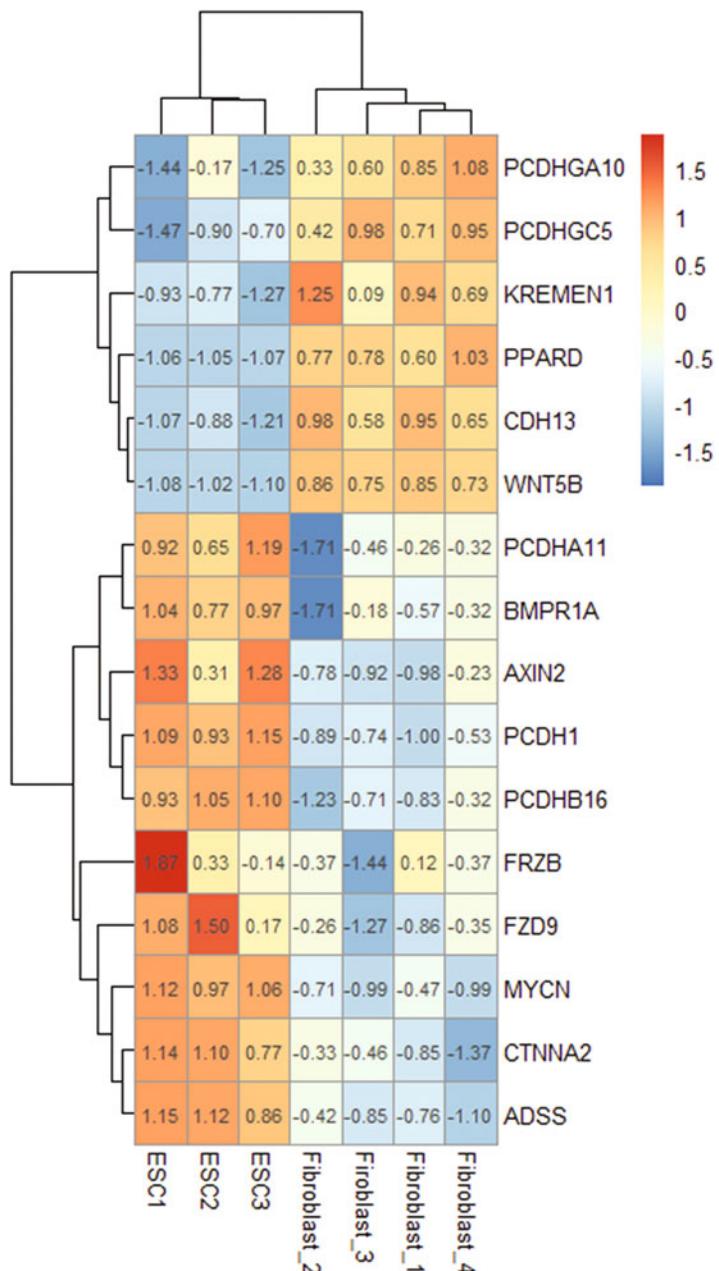


Fig. 6 The same heat map as in Fig. 5 but with the cell width modified

We can separate the heat map into two panels based on clusters representing the two types of cells using the argument of *cutree_cols* (Fig. 8):

```
pheatmap(heatmap_log2, scale = "row", cellwidth = 22, cellheight = 20, display_numbers = TRUE,
        cutree_cols = 2)
```

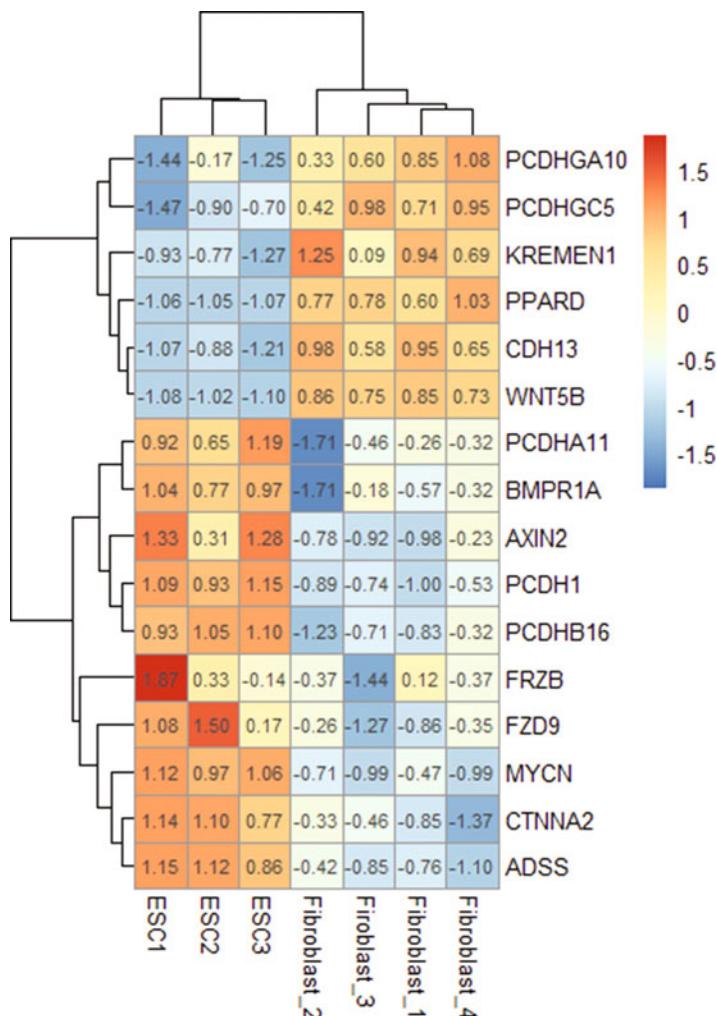


Fig. 7 The same heat map as in Fig. 6 except for additional modification in cell heights

We can include a heat map title using the argument of *main* (Fig. 9):

```
pheatmap(heatmap_log2, scale = "row", cellwidth = 22, cellheight = 20, display_numbers = TRUE,
cutree_cols = 2, main = "Differential expression of some genes in WNT pathway between human
fibroblasts and ESCs")
```

The heat map title is too wide compared with that of the heat map image. We can split it into two or three lines using the *paste* function along with the new line operator \n (Fig. 10):

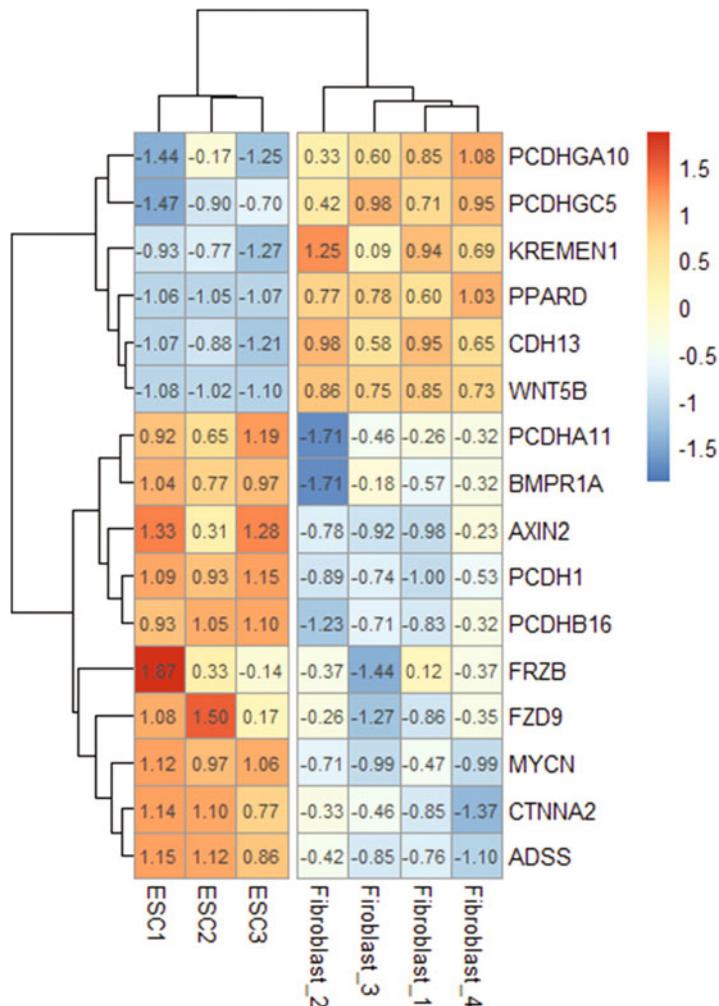


Fig. 8 The same heat map as in Fig. 7 except for a gap introduced to separate panels for the two cell types

```
pheatmap(heatmap_log2, scale = "row", cellwidth = 22, cellheight = 20, display_numbers = TRUE,
cutree_cols = 2, main = paste("Differential expression of", "\nsome genes in WNT pathway",
"\nbetween human fibroblasts and ESCs"))
```

We can change the font sizes for numbers and text in the heat map, using the arguments of *fontsize*, *fontsize_row*, *fontsize_col*, or *fontsize_number* for all, row names, column names, or numbers in the cells, respectively (Fig. 11):

Differential expression of some genes in WNT pathway between human fibroblasts and ESCs

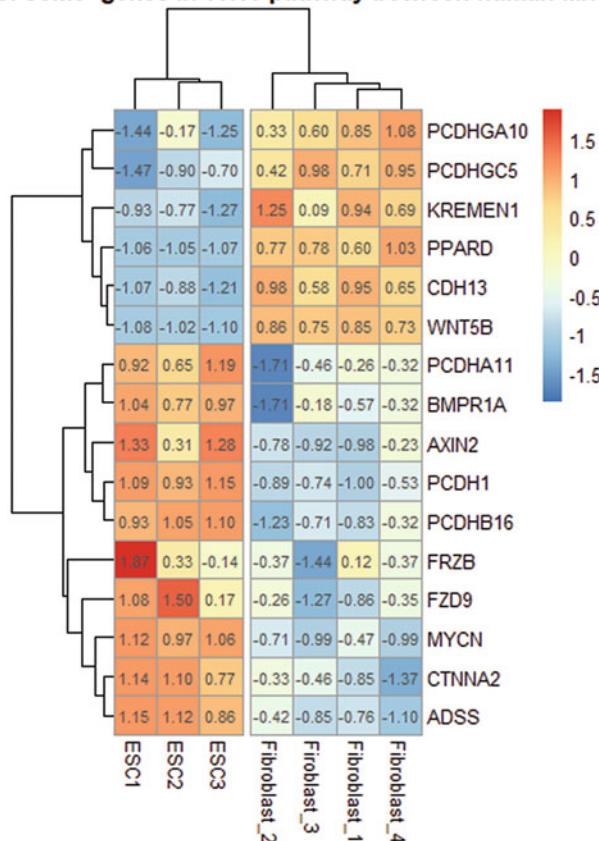


Fig. 9 The heat map in Fig. 8 except that a title is included

```
pheatmap(heatmap_log2, scale = "row", cellwidth = 22, cellheight = 20, display_numbers = TRUE,
cutree_cols = 2, main = paste("Differential expression of",
"some genes in WNT pathway",
"between human fibroblasts and ESCs"), fontsize = 14)
```

The default font size is 10. We found that the new font size for the numbers is too large for the cell size. We can change either the size of cell or the font size of numbers to make them matched. We redefine the cellwidth = 28 (see Fig. 12).

We can change the color of the cell border using the *border_color* argument (Fig. 13):

```
pheatmap(heatmap_log2, scale = "row", cellwidth = 28, cellheight = 20, display_numbers = TRUE,
cutree_cols = 2, fontsize = 14, border_color = "magenta")
```

You can try other colors by replacing magenta in the above script with white, black, grey, pink, yellow, cyan, green, and others.

**Differential expression of
some genes in WNT pathway
between human fibroblasts and ESCs**

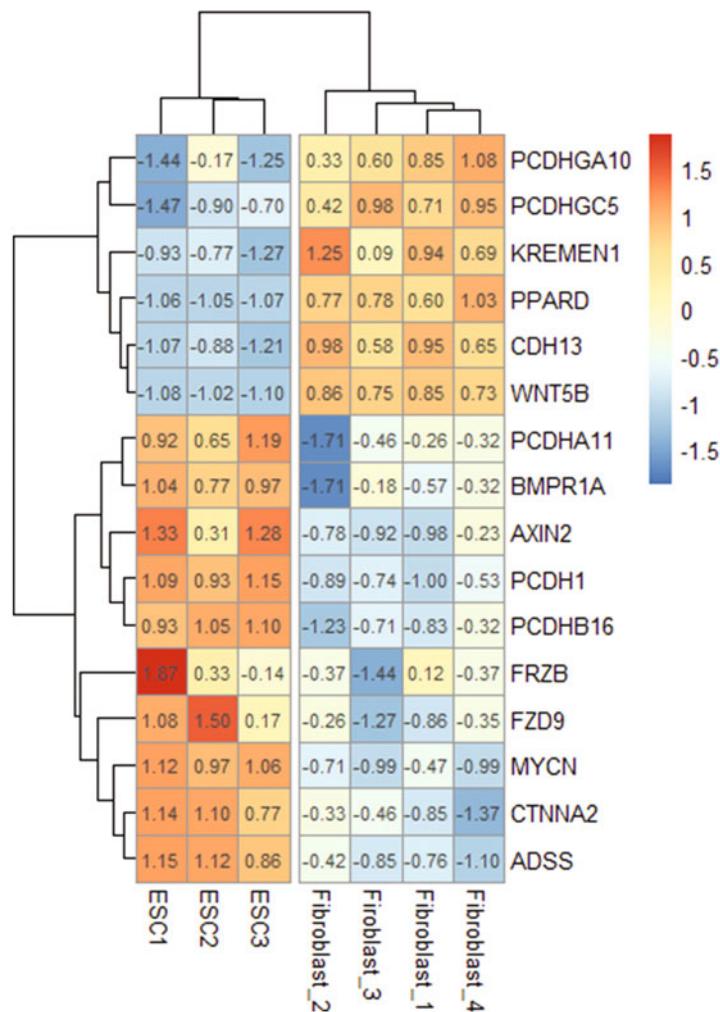


Fig. 10 The heatmap in Fig. 9 but with its title split in three lines

We can change the angle of the text for column labels using argument of *angle_col* (Fig. 14) (Please note that only five predefined angles are available, 0, 45, 90, 270, and 315):

```
heatmap(heatmap_log2, scale = "row", cellwidth = 28, cellheight = 20, display_numbers = TRUE,
       cutree_cols = 2, fontsize = 14, border_color = "magenta", main = paste("Differential expression of",
       "\nsome genes in WNT pathway", "\nbetween human fibroblasts and ESCs"), angle_col = "45")
```

Differential expression of some genes in WNT pathway between human fibroblasts and ESCs

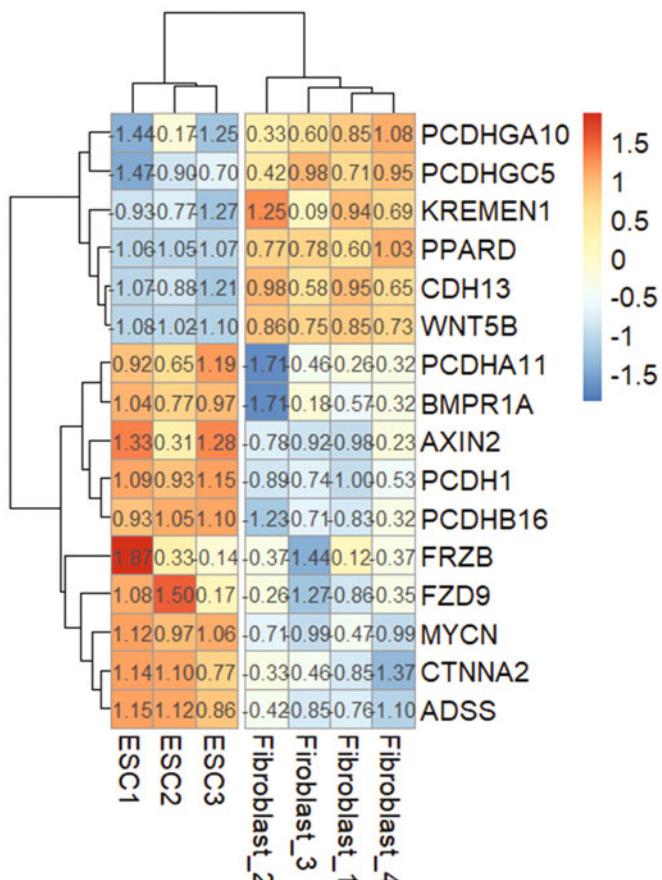


Fig. 11 The same heat map as in Fig. 10 but with a larger font size

We can change the height of the dendrogram using arguments of `treeheight_row`, and/or `treeheight_col` (Fig. 15):

```
heatmap(heatmap_log2, scale = "row", cellwidth = 28, cellheight = 20, display_numbers = TRUE,
cutree_cols = 2, fontsize = 14, border_color = "magenta", main = paste("Differential expression of",
"\n some genes in WNT pathway", "\nbetween human fibroblasts and ESCs"), treeheight_row = 200,
treeheight_col = 100)
```

If you do not want the tree to show up in the image, the `treeheight` value(s) can be set to 0 (Fig. 16).

```
heatmap(heatmap_log2, scale = "row", cellwidth = 28, cellheight = 20, display_numbers = TRUE,
cutree_cols = 2, fontsize = 14, border_color = "magenta", main = paste("Differential expression of",
"\n some genes in WNT pathway", "\nbetween human fibroblasts and ESCs"), treeheight_row = 0,
treeheight_col = 0)
```

Differential expression of some genes in WNT pathway between human fibroblasts and ESCs

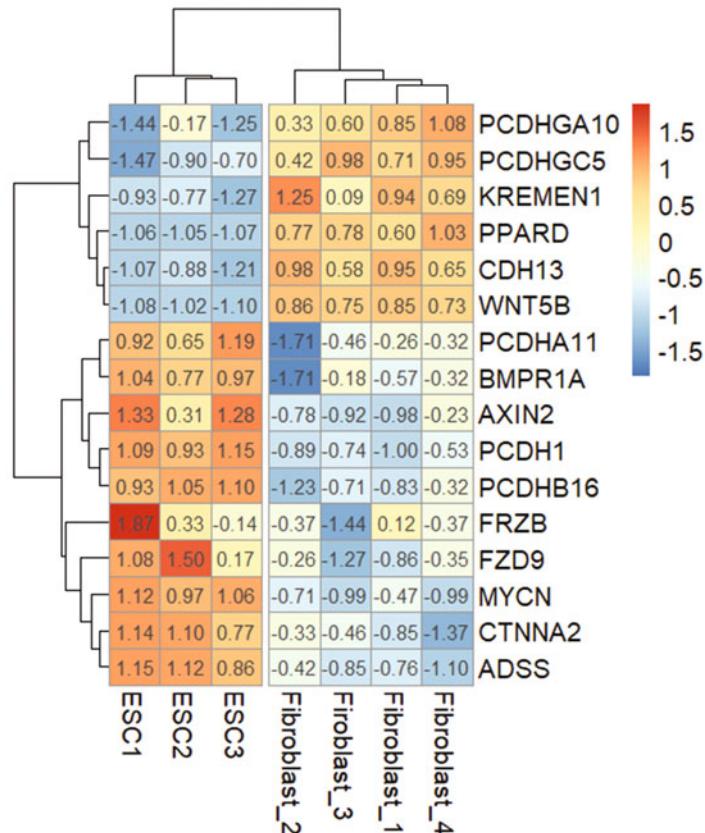


Fig. 12 The same heat map as in Fig. 11 but with the cell width increased to accommodate the larger font size

You may just need heat map and will add title, gene names, sample names later in Illustrator to produce a high-quality presentation. To this end, you can remove the *main* argument, and set *show_rownames*, *show_colnames*, and *display_numbers* to F or FALSE (Fig. 17).

```
pheatmap(heatmap_log2, scale = "row", cellwidth = 28, cellheight = 20, cutree_cols = 2, fontsize = 14,
border_color = "magenta", treeheight_row = 0, treeheight_col = 0, display_numbers = FALSE,
show_rownames = F, show_colnames = F)
```

We can give the rows or columns specific names rather than row names and column names in the original matrix using the arguments of *label_col* and/or *label_row*. In this case, we need to set *show_rownames* or *show_colnames* to T or TRUE, or just remove these two arguments in order to display the new names (Fig. 18):

Differential expression of some genes in WNT pathway between human fibroblasts and ESCs

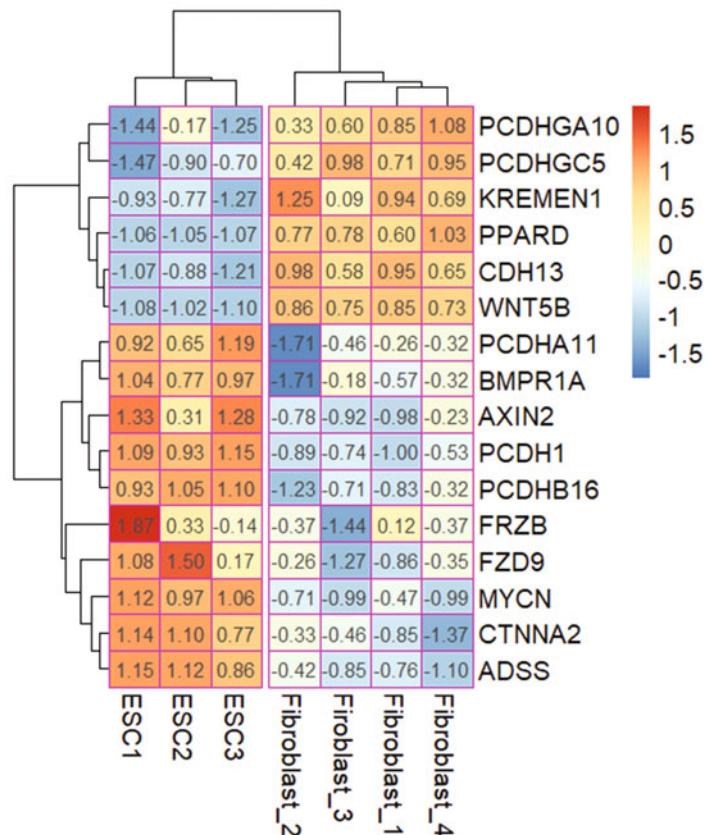


Fig. 13 The same heat map as in Fig. 12 but with magenta cell borders

```
pheatmap(heatmap_log2, scale = "row", cellwidth = 28, cellheight = 20, display_numbers = FALSE,
cutree_cols = 2, fontsize = 14, border_color = "magenta", treeheight_row = 0, treeheight_col = 0,
labels_col = c("F1", "F2", "F3", "F4", "PSC1", "PSC2", "PSC3"))
```

We can use a color palette rather than the default one. For this purpose, we will load the color library RColorBrewer [9] using the *library()* function, or by checking the box next to RColorBrewer in the Packages window.

```
library(RcolorBrewer)
```

To know what color palettes RColorBrewer includes, use the following R command:

```
display.brewer.all() # The RcolorBrewer palettes appear in Plots window.
```

Differential expression of some genes in WNT pathway between human fibroblasts and ESCs

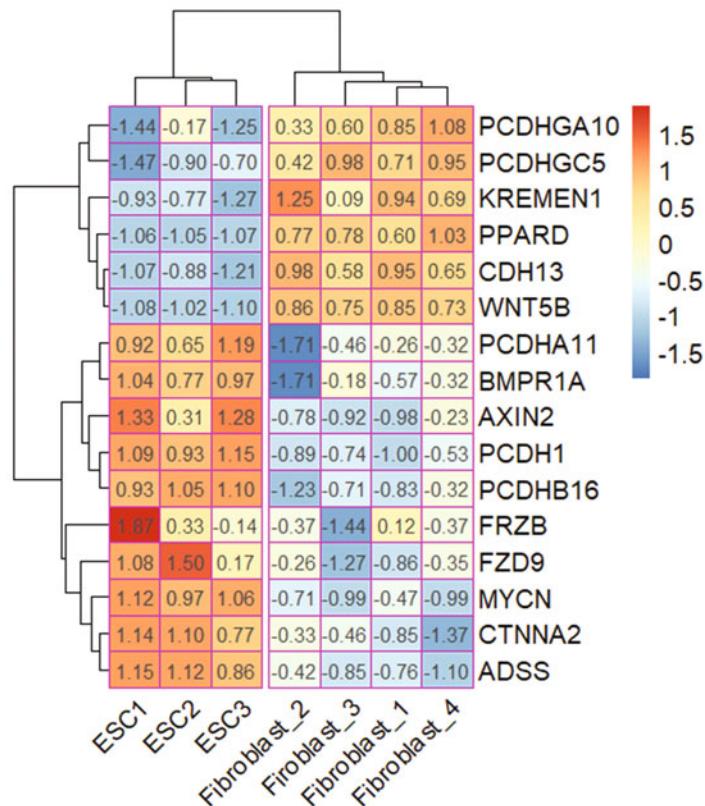


Fig. 14 The same heat map as in Fig. 13 but with its column names tilted

For scientific publications, more and more journals encourage the authors to use color-blind friendly palettes. To find out which color palettes are color-blind friendly, use the following script:

```
display.brewer.all(colorblindFriendly = T)      # All colorblind friendly palettes appear in the Plots window.
```

Now, you can test another color-blind diverging palettes PuOr using the following R script (Fig. 19):

```
heatmap(heatmap_log2, scale = "row", cellwidth = 28, cellheight = 20 ,  
fontsize = 14, border_color = "magenta", treeheight_row = 0,  
treeheight_col = 0, angle_col = "45", color = brewer.pal(11, "PuOr"))
```

The resolution looks good as well with the new color palette (Fig. 19).

**Differential expression of
some genes in WNT pathway
between human fibroblasts and ESCs**

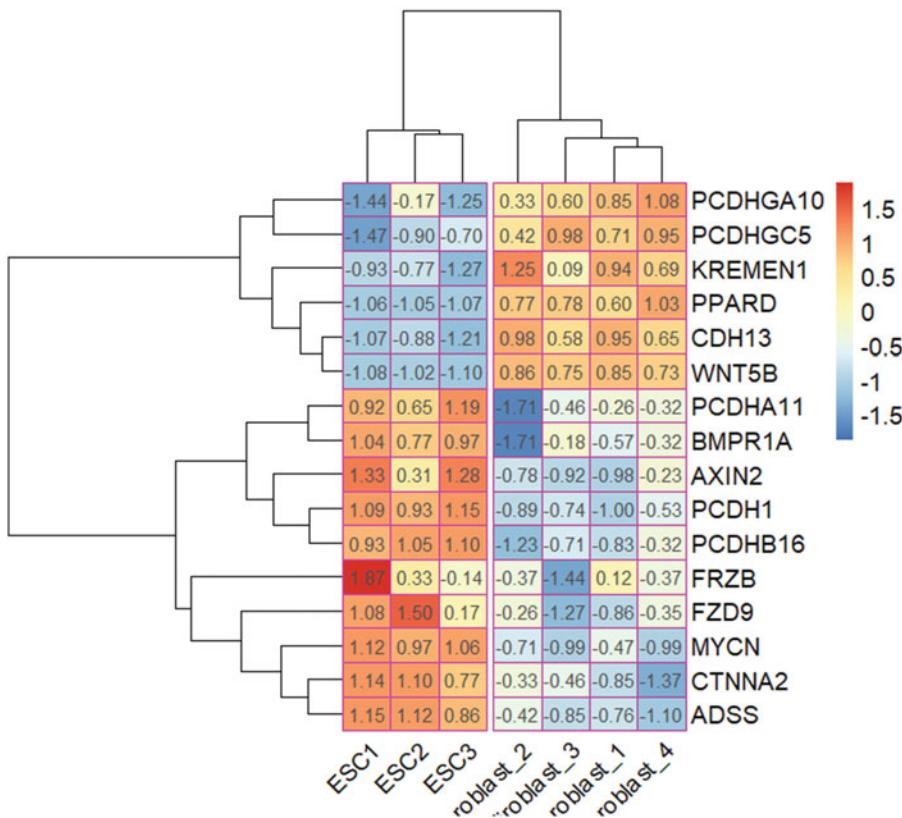


Fig. 15 The same heat map as in Fig. 14 but with increased heights of trees

Try not to scale the rows with the color palette *PuOr* (Fig. 20):

```
pheatmap(heatmap_log2, cellwidth = 28, cellheight = 20, fontsize = 14, border_color = "magenta",
treeheight_row = 0, treeheight_col = 0, angle_col = "45", color = brewer.pal(11, "PuOr"), scale =
"none")
```

The differentiation power has decreased without scaling (Fig. 20).

Try to use the original normalized read counts for a heat map with the new colors without log2 transformation (Fig. 21).

```
pheatmap(cellwidth = 28, cellheight = 20, fontsize = 14, border_color = "magenta", treeheight_row = 0,
treeheight_col = 0, angle_col = "45", color = brewer.pal(11, "PuOr"), my1stHeatmap1)
```

The differentiation power becomes even worse without log2 transformation and scaling (Fig. 21).

Differential expression of some genes in WNT pathway between human fibroblasts and ESCs

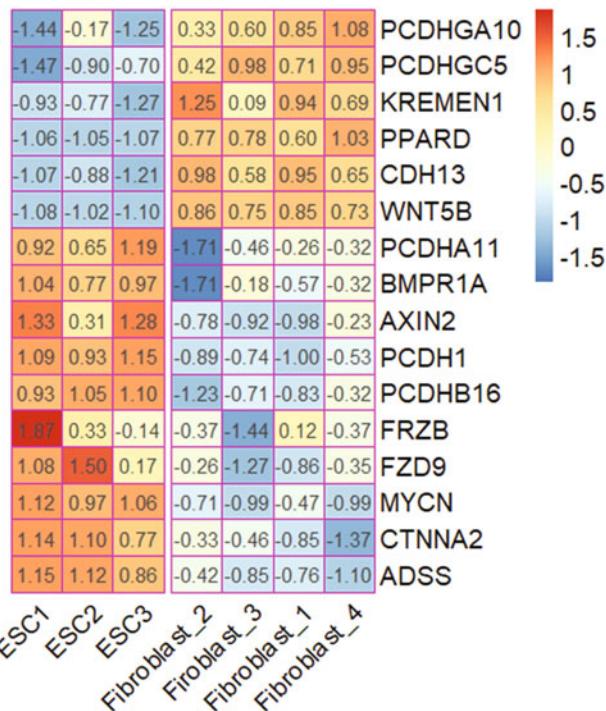


Fig. 16 The same heat map as in Fig. 15 but with its dendograms removed

2.9 Timing

For a novice without any prior R experience, it takes one day to become competent in generating heat maps using the procedure provided in this protocol. After being proficient, one can generate a heat map from a read count table in less than 30 min.

2.10 Troubleshooting

Troubleshooting advices can be found in Table 2. For additional R experience and skills, audiences can use my recent tutorial about generating boxplots and violin plots using R [10].

2.11 Anticipated Results

At the end of the morning practice, audiences will download and install R, as well as RStudio on to his/her own computers. The audiences will then become familiar, by hands-on experience, with some basic R concepts, commands, functions, and some scripts including some basic manipulation of tables, which will help the audiences to use the demo table for preparation of RNA-seq data to generate their own heat maps.

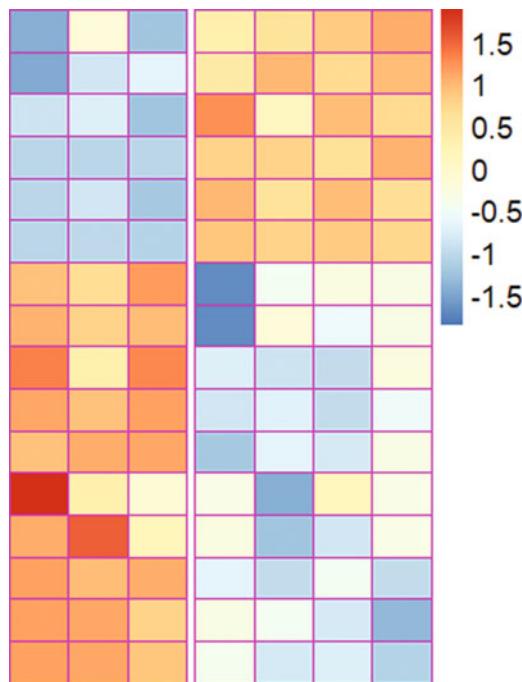


Fig. 17 The same heat map as in Fig. 16 but with all of its labels removed

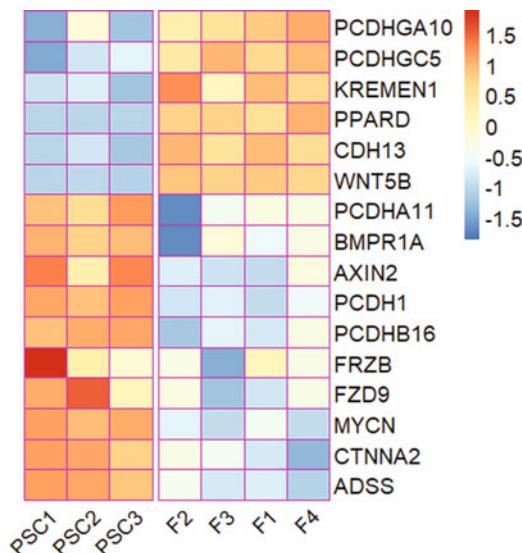


Fig. 18 Heat map with customized column labels

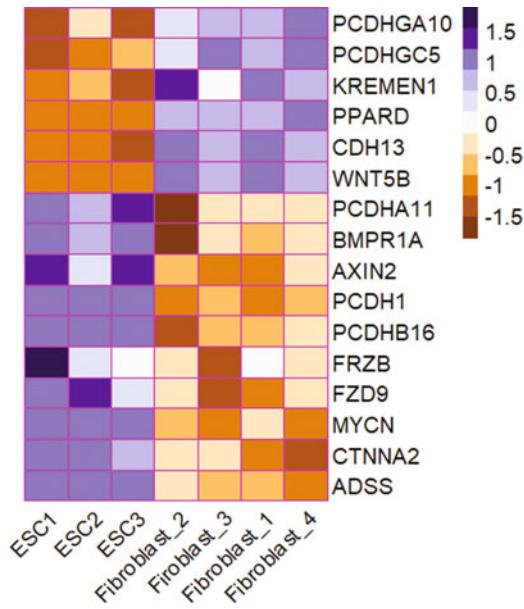


Fig. 19 Heat map for log2-transformed read counts and row scaling, with a color palette (PuOr) different from the default

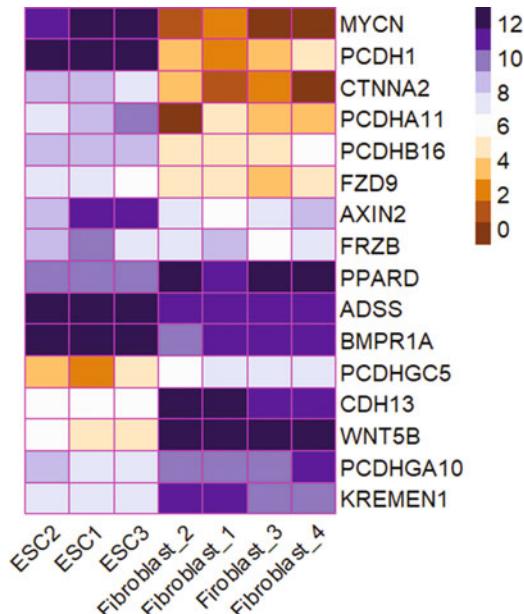


Fig. 20 A low-resolution heat map without scaling. The same heat map as in Fig. 19, but without row scaling. Similar to Fig. 2, but with different colors

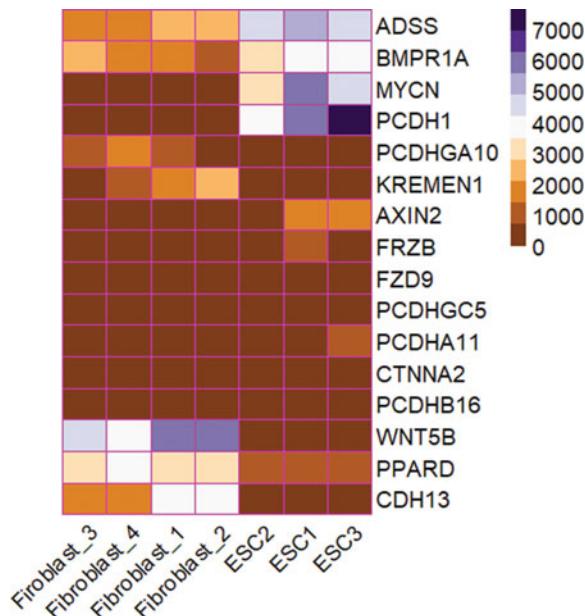


Fig. 21 A heat map with very low differentiation power. The same heat map as in Fig. 19, but with the original read counts and without scaling. Similar to Fig. 1, but with different colors

At the end of the day, audiences will be able to generate heat maps using data frame of RNA-seq read counts. The audiences will also become proficient in manipulating the resulting heat maps for a desired size and appearance.

Acknowledgments

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Table 2
Troubleshooting table

Problems	Possible causes	Potential solutions
Cannot download R or Rstudio	Different computer systems and browsers used.	Downloading and installation is generally easy. Just follow instruction on your computer rather than steps here. If problems persist, ask your IT personnel for help.
RStudio not open after installation	Computer system is too old.	Download and install earlier version of RStudio that can run on your computer.
R does not read my read count table	The format may be wrong. The file extension is missing.	Save the table as .csv format. Check if the file extension .csv is missing.
Could not find the function <i>pheatmap</i>	<i>pheatmap</i> package is not installed; <i>pheatmap</i> is not loaded.	Install <i>pheatmap</i> ; load <i>pheatmap</i>
Heat map fails with warning: NA/Nan/Inf in function call	The read count table contain characters: NA (not available, NaN (not a number), or Inf.	Replace NA, NaN, or Inf with appropriate numeric numbers.
<i>pheatmap</i> does not work with a warning: object “yourobjectname” not found.	Object names in R are case sensitive, and your input may use the wrong cases.	Check if the object name (matrix name) to be heat mapped is correctly spelled. Pay attention to capital letters.
Heat map does not save with a warning: object “yourfilename” not found.	File name is not enclosed in quotation marks.	Enclose your heat map file name with quotation marks.
Heat map does not save with a warning: improper filename	The extension of the file name is missing.	Add file format extension such as .pdf, .png, or .tiff
Heat map does not show in plots Window after saving previous heat maps	R is using other graphics device.	Run <i>dev.off()</i> one more time. It will work when you see “null device” after running <i>dev.off()</i> .
Heat map fails with a warning: object “true” not found	The logical objects TRUE or FALSE should be capitalized.	Change “true” to “TRUE” or “T”. This is true for “false”.
Heat map fails with a warning: unexpected “=”	When use two-part object such as <i>display_numbers</i> , a hyphen is used instead of an underscore.	Change the hyphen sign to an underscore sign.
Heat map fails with a warning: could not find function “brewer.pal”	The RColorBrewer is not loaded.	Load the color package RColorBrewer.
RColorBrewer will not load	Package name is not spelled correctly.	Use capital R and B in RColorBrewer, or just click the box next to RcolorBrewer in the Packages window.

(continued)

Table 2
(continued)

Problems	Possible causes	Potential solutions
Heat map fails when use new color with a warning: not a valid palette name for brewer.pal	The color object is not spelled correctly.	Check the color object name and make sure appropriate uppercase letters are used.
Cannot sort using the <i>order()</i> function	The comma is missing.	Add a comma “,” within the index operator [].
Customized labels for columns are with the wrong samples	The labels in the character vector for the argument <i>labels_col</i> is in the wrong order.	Order the labels in the same order as they appear in the matrix columns.

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