

Chapter 2

Induced Pluripotent Stem Cells (iPSCs): An Emerging Model System for the Study of Human Neurotoxicology

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Abstract

This chapter describes the materials and methods necessary to generate human induced pluripotent stem cells (iPSCs) from primary human fibroblasts and direct their differentiation into neural progenitor cells. Application of such methods is an emerging model for the study of neurotoxicity focused on human neurons and glia derived from specific patients. The techniques described here include primary human fibroblast culture, lentiviral/retroviral-mediated iPSC inductions, iPSC clonal expansion and maintenance, validation of pluripotency markers, and neuronal differentiation of iPSCs. Methods and applications using iPSCs are rapidly changing: here we describe the current methods used in our laboratories. The iPSC induction method featured in this chapter is based on a two-step viral transduction approach described by Dr. Shinya Yamanaka and colleagues (Cell 131:861–872, 2007) modified following the protocol of Dr. Sheng Ding and collaborators (Nat Methods 6:805–808, 2009). The neuralization method featured in this chapter is based on the method described by Lorenz Studer and colleagues (Nat Biotechnol 27:275–280, 2009). Maintenance and cryostorage methods were developed in our lab by optimizing a combination of approaches described in the literature. This chapter is not meant to be comprehensive, but instead focuses on the core competencies needed to begin working with human iPSCs and neuralization of these cells for toxicological studies.

Key words: Induced pluripotent stem cells, Human models of neurotoxicity, Neuronal differentiation, Patient-derived fibroblasts

1. Introduction

A major breakthrough was announced in 2007 by two independent labs, headed by Drs. Shinya Yamanaka and James Thomson, reporting the generation of human induced pluripotent stem cells (iPSCs) from adult human dermal cells with a developmental potential seemingly equivalent to human embryonic stem cells (hES cells) (1, 2). iPSCs can generate cells from all three embryonic germ layers, and thus are very similar to hES cells (1–3). Because of these traits,

iPSCs open a new and exciting branch of human stem cell based toxicological and disease research. The study of neurotoxicology represents an almost ideal application of this technology given the complexities of Human diseases with diverse genetic and environmental risk factors. Furthermore, the ability to differentiate neurons and glia from patient iPSCs offers a chance to examine in detail changes in both the development and maintenance of neural function resulting from complex genetic inheritance patterns. Finally, the relative ease to expand, maintain, culture, and differentiate iPSCs will enable utilization of this resource by a broader range of laboratories around the world, expanding both the scope and depth of research into human disease.

Environmental stressors are thought to interact with an individual's genetic makeup to influence or cause neurological disease. These toxicological actions may occur at any point during development as well as later during adulthood. The multigenic inheritance pattern of environmental susceptibility makes it difficult to evaluate the risk that environmental agents pose for specific diseases or persons. The advent of iPSC technology will enable researchers to generate in vitro cultures of patient-specific neurons to assess patient-specific susceptibility to neurotoxins. This technology opens the opportunity to characterize the cellular, physiological, pharmacological, and molecular properties of living human neurons with identical genetic determinants as individuals with neurological disease (Fig. 1). Furthermore, if a comprehensive clinical history of the patients is known, iPSC-based studies can compare patient populations showing sensitivity to particular environmental agents, divergent clinical outcomes, and differing co-morbidities. Thus, iPSC technology has the potential to revolutionize scientific approaches to human health.

The application of human pluripotent stem cell technology to toxicological risk assessment posits that cells differentiated from patient-derived stem cells will model the toxicant sensitivity of the patient's own cells. A number of technical and theoretical hurdles need to be overcome before the utility of this approach can be realized. For example, efficiency and consistency of stem cell derivation; contribution of epigenetic changes; and protocols for differentiation, exposure paradigms, and assessment of toxicity need to be optimized. Perhaps though the most fundamental issue that must be addressed soon is whether the methods for generating patient-derived pluripotent stem cells are capable of yielding a consistent model of sensitivity to environmental toxicants. Several groups, including our own, are currently performing research and development to validate iPSCs as a model of human toxicological risk.

This chapter describes current methods to generate and validate iPSCs using the basic method developed by Yamanaka and colleagues of lentiviral transduction of fibroblasts to allow expression of the mouse retroviral receptor, *Slc7a1*, followed by mouse

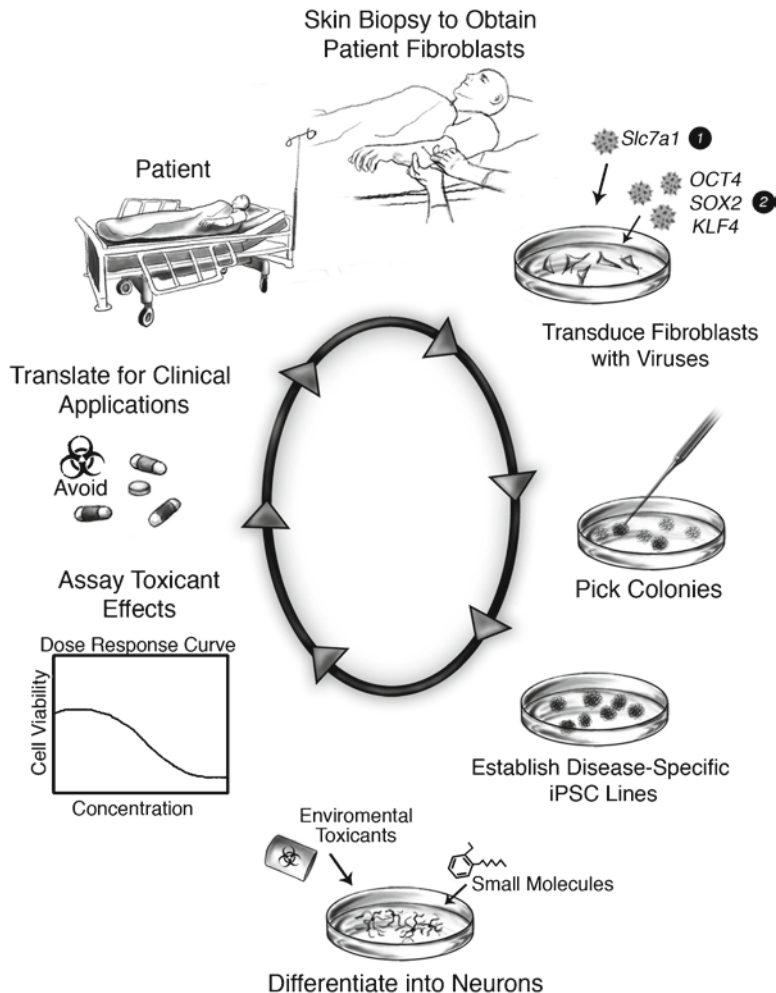


Fig. 1. Scientific approach: from bedside to bench and back again. A schematic overview is given of the steps involved in deriving patient-specific neurons and how observations from experimentation with these neurons can potentially benefit patients. Fibroblasts harvested from a skin biopsy are transduced to become patient-specific human induced pluripotent stem cells (iPSCs). The human iPSCs are then differentiated into neurons that can be assessed for their response to the neurotoxicants of interest. The knowledge obtained from these experiments may directly benefit the patients or allow the testing of novel hypotheses related to disease pathogenesis.

ecotropic retroviral transduction with the three genes shown to be sufficient for induction of pluripotency, *SOX2*, *KLF4*, and *OCT3/4* (1, 4). We provide detailed methods from our laboratory's adaptation of a variety of available methods. The topics covered include culturing of primary human fibroblasts from skin biopsies, preparation of virus for transductions, induction of pluripotency in human fibroblasts, isolation of human iPSC colonies, basic maintenance and expansion of human iPSC lines, and a discussion of first step validation protocols to confirm the human

iPSC phenotype. Finally, we discuss methods we have adapted from the work of Lorenz Studer and colleagues to induce neural progenitors from human iPSCs (5). Throughout this chapter, we refer the reader to key publications that offer alternative methods or expanded approaches in working with human iPSCs.

2. Materials

The selection of basic materials for iPSC culture and differentiation is critical for success. Indeed, for many of the reagents it is highly recommended that these specific vendors and catalog numbers be utilized. A list of antibodies, including dilutions and vendor information, are provided in Table 1. A complete list of the stock reagents needed, including catalog number and vendor name, is provided in Table 2. Cell culture conditions for all procedures in this chapter utilize a standard tissue culture incubator, with a 5% CO₂ humidified environment at 37°C. Due to the use of viruses and primary human cells, human iPSC cultures and human fibroblast reprogramming should be carried out in a biosafety level 2 (BSL2) approved facility. Below we provide the recipes for all of the media and solutions described in the methods Sect. 3.

Volume	Component	Final concentration
<i>Complete DMEM media</i>		
440 mL	DMEM (Mediatech, Inc. #10-013-LV)	
50 mL	Fetal Bovine Serum (FBS) (Sigma # F2442)	10%
5 mL	Pen/Strep (100×, Mediatech # 30-002-CI)	1×
5 mL	Non-essential amino acids (100×, Sigma # M7145)	1×
<i>hES media</i>		
385 mL	DMEM/F12 (Invitrogen #11330-032)	
100 mL	Knockout Serum Replacement (Invitrogen #10828)	20%
2.5 mL	Glutamax (100×, Invitrogen #35050-061)	2 mM
5 mL	Pen/Strep (100×, Mediatech # 30-002-CI)	1×
5 mL	Non-essential amino acids (100×, Sigma # M7145)	1×
0.2 mL	rhFGF, basic (20 µg/mL in D-PBS, Promega # G5071)	8 ng/mL
3.5 µL	β-Mercaptoethanol (Sigma # M3148)	100 µM
<i>Neuralization media</i>		
410 mL	Knockout DMEM (Invitrogen #12660)	
75 mL	Knockout Serum Replacement (Invitrogen #10828)	15%
5 mL	Glutamax (100×, Invitrogen #35050-061)	2 mM
5 mL	Pen/Strep (100×, Mediatech #30-002-CI)	1×
5 mL	Non-essential amino acids (100×, Invitrogen # M7145)	1×
1.93 µL	β-Mercaptoethanol (Sigma # M3148)	55 µM

<i>Fibroblast freezing solution</i>		
12 mL	DMEM (Mediatech, Inc. #10-013-LV)	60%
6 mL	FBS (Sigma # F2442)	30%
2 mL	DMSO (Sigma # D8418)	10%
<i>Feeder-free iPSC freezing solution</i>		
9 mL	mTeSR1 (StemCell Technologies. Item#05850)	90%
10 μ L	Rho Associated Kinase (ROCK) inhibitor Y-27632 (1,000 $\times$ in H ₂ O, Tocris Bioscience #1254)	10 μ M
1 mL	DMSO (Sigma # D8418)	10%
<i>CTK solution</i>		
5 mL	2.5% (w/v) trypsin (Invitrogen #15090-046)	0.25%
5 mL	1 mg/mL collagenase IV (Invitrogen #17104-019) (in D-PBS)	0.1 mg/mL
0.5 mL	0.1 M CaCl ₂ in cell culture grade H ₂ O (Sigma # C7902)	1 mM
10 mL	Knockout Serum Replacement (Invitrogen #10828)	20%
30 mL	Cell culture grade H ₂ O (Invitrogen #15230-147)	

Table 1
Primary and secondary antibodies

	Dilution	Source and item number
<i>Primary antibodies</i>		
TRA-1-60	1:100	Millipore. Item# MAB4360
CD57/HNK-1	1:100	Sigma. Item# C6680
NESTIN	1:200	Millipore. Item# AB5922
OCT4	1:200	Cell Signaling Technology. Item# 2750
PAX6	1:200	Covance. Item# PRB-278P
SSEA3 (MC-631)	1:100	Millipore. Item# MAB4303
SSEA4 (MC-813-70)	1:200	Dev. Studies Hyb Bank. Item# MC-813-70
SSEA4-Alexa Fluor 488	1:100	BD Pharmingen. Item# 560308
TRA-1-60-Alexa Fluor 555	1:25–50	BD Pharmingen. Item# 560121
TRA-1-81-Dylight 488	100	Stemgent. Item# 09-0069
<i>Secondary antibodies</i>		
DyLight 649 Affinity Purified Donkey Anti-Mouse IgG (H+L)	1:400	Jackson ImmunoResearch Laboratories. Item# 715-495-150
DyLight 649 Affinity Purified Donkey Anti-Rabbit IgG (H+L)	1:400	Jackson ImmunoResearch Laboratories. Item# 711-495-152
DyLight 549 Affinity Purified Donkey Anti-Mouse IgG (H+L)	1:800	Jackson ImmunoResearch Laboratories. Item# 715-505-150
DyLight 549 Affinity Purified Donkey Anti-Rabbit IgG (H+L)	1:800	Jackson ImmunoResearch Laboratories. Item# 711-505-152
DyLight 649 Affinity Purified Goat Anti-Rat IgM, μ chain specific	1:400	Jackson ImmunoResearch Laboratories. Item# 112-495-020

Table 2
Materials and reagents

Reagent	Source and item number
293-FT cell line	Invitrogen. Item# R700-07
Accutase	Innovative Cell Technologies. Item# AT-104
BD Matrigel™ hESC-qualified Matrix	BD Biosciences. Item# 354277
Blasticidin S	Invitrogen. Item# R210-01
Dispase	StemCell Technologies. Item# 07923
D-PBS	Mediatech. Item# 21-031-CV
Normal donkey serum	Jackson ImmunoResearch Laboratories. Item# 017-000-121
Fugene 6 transfection reagent	Roche Applied Science. Item# 11814443001
Geneticin (G418)	Mediatech. Item# MT61234RG
Hoechst (bisBenzimide H 33258)	Sigma. Item# B1155
Leukocyte Alkaline Phosphatase Kit	Sigma. Item# 85L1-1KT
LIF (leukemia inhibitory factor)	Millipore. Item# LIF1010
Lipofectamine 2000 transfection	Invitrogen. Item# 11668-019
Mitomycin-C	Sigma. Item# M4287
mTeSR1 medium	StemCell Technologies. Item# 05850
Noggin	R&D Systems. Item# 719-NG
OPTI-MEM I reduced-serum medium	Invitrogen Corporation. Item# 11058-021
16% Paraformaldehyde solution	Electron Microscopy Sciences Item# 15710
PBS (10× solution)	Mediatech Item# 46-013-CM
PD0325901	Cayman Item# 13034
PLAT-E cells	Cell Biolabs, Inc. Item# RV-101
pLenti6/UbC/mSlc7a1	Addgene. Plasmid 17224
pMXs-hc-MYC	Addgene. Plasmid 17220
pMXs-hKLF4	Addgene. Plasmid 17219
pMXs-hOCT3/4	Addgene. Plasmid 17217
pMXs-hSOX2	Addgene. Plasmid 17218
Polybrene	Millipore. Item# TR-1003-G
Puromycin	Sigma. Item# P8833
ROCK inhibitor, Y-27632	Tocris Bioscience. Item# 1254
SB431542	Stemgent. Item# 04-0010

(continued)

Table 2
(continued)

Reagent	Source and item number
SNL cells	MMRRC at University of California, Davis. Item# 015892-UCD
Thiazovivin	Stemgent # 04-0017
Triton X-100	Sigma. Item# T8787
Trypsin-EDTA (0.05%) 1×	Invitrogen Corporation. Item# 25300-054
ViraPower™ Bsd Lentiviral Support Kit	Invitrogen Corporation. Item# K4970-00

3. Methods

3.1. Procurement, Propagation, and Preservation of Human Fibroblasts

Human iPSCs are derived from human somatic cells by the transgenic expression of a small number of factors (see Sect. 3.3). Human adult dermal fibroblasts are widely used for the production of patient-specific iPSCs, but derivation of iPSCs from human keratinocytes has also been reported (6). Human fibroblast cultures can be established from skin biopsies or can be obtained from commercial sources. Organizations offering primary human fibroblasts include:

American Type Culture Collection, ATCC, <http://www.atcc.org>
Cell Applications, <http://www.cellapplications.com>

Coriell Institute for Medical Research, <http://ccr.coriell.org/>

European Collection of Cell Cultures, ECACC, <http://www.ecacc.org.uk>

Japanese Collection of Research Bioresources, <http://cellbank.nibio.go.jp>

Lonza, <http://www.lonza.com/group/en.html>

Riken Biosource Center, <http://www.brc.riken.jp>

3.1.1. Obtaining Fibroblasts from a Human Skin Biopsy

To obtain patient-specific tissue, we perform 3 mm punch skin biopsies using an Acu Punch (Acuderm, Inc). The removed tissue is cultured under standard culture conditions and after 3–7 days, fibroblasts migrating out from the skin punch can be observed (Fig. 2). Two weeks later these fibroblasts can be passaged for derivation of iPSCs and cryostocks generated for future use.

3.1.1.1. Procedure

1. Skin biopsies should be obtained only with Institutional Review Board approval.
2. Obtain skin biopsies (about 3 mm punch biopsy) from patients using standard sterile methods.

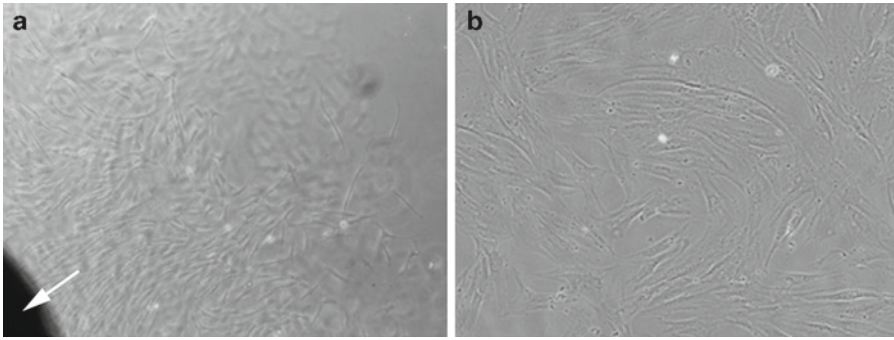


Fig. 2. Procurement of human fibroblasts. (a) The tissue of a skin biopsy is transferred into a cell culture dish and maintained in complete DMEM media. Over the next 2 weeks, fibroblasts migrate out of the tissue and proliferate in the dish. This is an image taken 11 days after the biopsy was performed. Fibroblasts have migrated away from the skin tissue (*arrow*). After about 2 weeks, the fibroblasts are harvested and are ready for propagation. (b) The fibroblasts shown were harvested from the same biopsy tissue seen in (a). These fibroblasts have been passaged three times and are shown here at about 80% confluency. At this point the human fibroblasts are ready for another passage or can be used for iPSC generation.

3. The tissue is transferred to a 35 mm dish containing 1 mL complete DMEM media.
4. Skin sample is transferred to a tissue culture hood and halved with a scalpel or sterile scissors (sterilized tools should be used for tissue from each patient to prevent any cross-contamination).
5. Put each half of the biopsy tissue into a well of a 6-well plate containing 0.5 mL of complete DMEM media. It is important that the tissue makes contact with the dish surface at this stage; if necessary a sterile glass cover slip (22 × 22 mm) can be put on top of the skin tissue for additional weight to ensure contact between the tissue and the culture dish surface.
6. When the tissue is in contact with the culture dish surface, slowly add 1.5 mL of complete DMEM media.
7. Move dish carefully into a standard tissue culture incubator (5% CO₂, humidified environment at 37°C).
8. Aspirate media and add additional 2 mL complete DMEM media 48 h later.
9. Feed cultures every 3–4 days with complete DMEM media.
10. Fibroblasts will migrate out of the biopsied tissue and are ready for propagation 1–2 weeks later.

3.1.2. Propagation of Human Fibroblasts

Human fibroblasts are best split when they reach about 80% confluency (Fig. 2b). The first passage is performed on fibroblasts growing in 6-well plates from which they are transferred into 10 cm dishes for further propagation. Volumes of media and enzyme needed for the first (when the cells are in 6-well plates) and later

propagations (when the fibroblasts are grown in 10 cm dishes) are both indicated, with the latter appearing in parentheses.

3.1.2.1. Procedure

1. Before the first passage move the cover slip (if present) into an empty well of the dish with the side that had faced the tissue up and remove the biopsied tissue punch from the well.
2. Wash fibroblasts and the cover slip two times with 2.5 mL (10 mL) of D-PBS to remove all media and serum.
3. Incubate cells and cover slip (present only for the first propagation) each with 1 mL (1 mL) of 0.05% trypsin-EDTA for about 5 min (check under the microscope for progress starting at 3 min).
4. Stop digestion by adding 2 mL (10 mL) of complete DMEM media.
5. Harvest fibroblasts and transfer cells from 1-well (dish) each into a 15 mL (50 mL) conical tube.
6. Add another 2.5 mL (10 mL) of complete DMEM media into each well and repeat the washing step.
7. Add solution containing trypsinized cells to conical tube from previous step.
8. Spin cells at $200 \times g$ for 5 min at room temperature.
9. Aspirate supernatant and resuspend cell pellet.
10. First passage only – resuspend cell pellet from each well of the 6-well plate in 10 mL complete DMEM media and plate cells into 10 cm dish.
11. Later passages only – resuspend cell pellets obtained from each 10 cm dish in 30–40 mL of complete DMEM media and plate cells into three to four new 10 cm dishes.
12. Label each culture with date, fibroblast identity, and passage number (see Note 1).
13. Feed cells with complete DMEM media every other day.

3.1.3. Freezing and Thawing of Human Fibroblasts

3.1.3.1. Procedure

Derivation of human iPSCs is most likely to be successful if attempted with early passage fibroblasts (ideally less than passage 5). Thus, early passage human fibroblasts should be frozen for future iPSC inductions should your first attempts not meet with your expectations.

Freezing of Human Fibroblasts

1. Proceed with steps 2–9 as described previously (Sect. 3.1.2).
2. Resuspend cell pellets in 3–4 mL of fibroblast freezing solution (usually we freeze at a concentration of about 2×10^5 cells/mL).
3. Pipette 1 mL of cell suspension into 1.5 mL Nalgene cryogenic vials (#5000-1020).

4. Put cryo-vials into a Nalgene cell culture cryofreezing container for overnight freezing at -80°C .
5. Transfer the cells into the liquid nitrogen storage facility the next day.

Thawing of Human Fibroblasts

1. Rapidly transfer the cryo-vial containing the fibroblasts from the liquid nitrogen storage into a 37°C water bath.
2. Thaw the cells by gentle agitation until most, but not all, of the contents is thawed.
3. Remove the vial from the water bath and let the remainder of the cells thaw.
4. Rinse the vial with 75% ethanol before transferring it into the tissue culture hood.
5. Transfer the thawed cell suspension into a 15 mL conical tube.
6. Slowly add 9 mL prewarmed complete DMEM media to the cells.
7. Spin the cells at $200\times g$ for 5 min at room temperature.
8. Resuspend the cell pellet (about 2×10^5 cells) in 10 mL of complete DMEM media.
9. Plate cells into a 10 cm dish.
10. Follow the growth and passage of the cells when they are about 80% confluent.

3.2. iPSC Induction: Lentivirus and Retrovirus Production

Reprogramming adult human fibroblasts into iPSCs is mediated by viral expression of transcription factors that are involved in maintaining the pluripotent state. The original report by Shinya Yamanaka's laboratory identified four transcription factors needed to reprogram cells: *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC* (1, 7, 8). We prefer to omit *c-MYC* from the transcription factor cocktail due to concerns of its potential to increase the number of partial iPSC colonies and its oncogenic character, as described by a subsequent paper from Yamanaka and colleagues (4).

Retroviruses containing the human cDNAs for *OCT3/4*, *SOX2*, and *KLF4* are used to drive expression of these genes in fibroblasts. For safety and efficiency reasons, ecotropic retroviruses are used. This approach requires the expression of the mouse membrane receptor protein gene, *Slc7a1*, for retroviral transduction of human fibroblasts. The *Slc7a1* gene is introduced into the fibroblasts by lentiviral transduction prior to retroviral transduction of the 3 factors. Lentiviruses are produced in the viral packaging cell line 293FT, while retroviruses are made in PLAT-E cells. Production of lentivirus requires co-transfection of pLenti6/UbC/mSlc7a1 with plasmids expressing the virus

packaging genes. Production of retrovirus in PLAT-E cells does not require co-transfection with additional viral packaging genes, as the cells stably express these factors already. Generation of retrovirus is done by transfection of single constructs encoding the genes for induction of pluripotency (pMXs-*h*, pMXs-*hKLF4*, pMXs-*hOCT3/4*, or pMXs-*hSOX2*). Viruses are obtained by collecting the supernatants from the transfected packaging cells. Retrovirus containing supernatant is pooled for transduction of the fibroblasts. Although some groups have reported freezing virus-containing supernatants at -80°C for later transduction, we prefer to use fresh virus-containing supernatants due to concerns of loss of viral titer resulting from the freeze–thaw cycle. The days marked in the timelines of this section correspond to those found in the timelines for fibroblast passaging and transduction (Sect. 3.3). All plasmids containing the cDNAs needed for virus production are available from Addgene and are listed in Table 2.

3.2.1. Passaging Packaging Cell Lines 293FT or PLAT-E

1. Aspirate the complete DMEM media from a 10-cm tissue culture dish when the cells have reached 80–90% confluency.
2. Wash once with 10 mL of D-PBS and aspirate.
3. Add 1 mL of 0.05% trypsin-EDTA and incubate at 37°C for 2 min.
4. Add 9 mL of complete DMEM media and gently pipette up and down to detach cells creating a single-cell suspension.
5. Place cells in a 15 mL conical tube and centrifuge at $200\times g$ for 5 min.
6. Aspirate media and resuspend the cell pellet in 2 mL complete DMEM media. For PLAT-E cells, add $1\text{ }\mu\text{g/mL}$ puromycin and $10\text{ }\mu\text{g/mL}$ blasticidin S to the media while expanding to maintain the expression of the viral packaging vector which contains resistance genes for these antibiotics. For 293FT cells, add 0.5 mg/mL G418 (geneticin) to the media.
7. Either propagate cells at a ratio of 1:4 to 1:6 for continued propagation or count the cells and dilute them to the appropriate concentration for transfection (4×10^6 per 10 cm dish for 293FT cells or 3.6×10^6 per 10 cm dish for PLAT-E cells) into complete DMEM media.

3.2.2. Transfecting 293FT Cells

1. One day prior to transfection (day 1), plate 4×10^6 293FT cells onto a 10-cm dish and incubate overnight in complete DMEM media *without* G418 in a standard tissue culture incubator.
2. The next day (day 2) pipette 1.5 mL of OPTI-MEM I media into a microcentrifuge tube, add $9\text{ }\mu\text{g}$ of ViraPower lentivirus packaging mix, and $3\text{ }\mu\text{g}$ of pLenti6/UbC plasmid DNA.

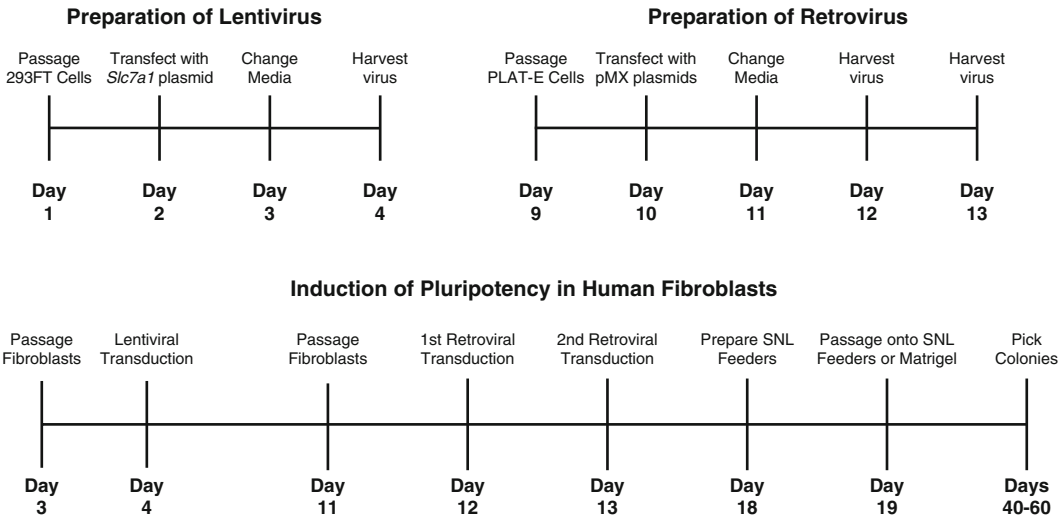


Fig. 3. Timeline for human iPSC induction. The induction of human fibroblast to become human iPSCs requires the preparation of several different agents (viruses and cells) at defined time intervals. The three timelines provided are set to the same schedule to allow the reader to organize the many steps required for the reprogramming of human fibroblasts.

3. In a separate tube, mix 1.5 mL of OPTI-MEM I with 36 μ L of Lipofectamine 2000 by finger tapping and incubate for 5 min at room temperature.
4. Combine the DNA- and Lipofectamine containing solutions, mix by finger tapping, and incubate for 20 min at room temperature.
5. Change the complete DMEM media on 293FT cells, then add transfection mixture to the cultures dropwise, and swirl gently.
6. Incubate the 293FT cells for 24 h.
7. Change media to fresh complete DMEM (day 3).

A timeline for generation of lentivirus for transduction using this protocol is shown in Fig. 3.

3.2.3. Transfecting
PLAT-E Cells

1. One day prior to transfection (day 9), seed three 10-cm dishes with 3.6×10^6 PLAT-E cells each (one for each pMX plasmid) and incubate overnight in complete DMEM media *without* puromycin or blasticidin S in a standard tissue culture incubator.
2. The following day (day 10), pipette 0.3 mL of OPTI-MEM I media into three microcentrifuge tubes, add 27 μ L of Fugene 6 transfection reagent to each, mix by finger tapping, and incubate for 5 min at room temperature.
3. Add 9 μ g of either *OCT3/4*, *KLF4*, or *SOX2* pMX plasmid to the three microcentrifuge tubes, mix by finger tapping, and incubate 15 min at room temperature.

4. Change the complete DMEM media on the PLAT-E cells, add transfection mixtures dropwise, one to each plate of PLAT-E cells, and swirl gently.
5. Incubate the PLAT-E cells for 24 h with transfection mixture (day 11).
6. Remove transfection mixture and replace with complete DMEM media.

A timeline for generation of retrovirus for transduction using this protocol is shown in Fig. 3.

3.2.4. Preparing the Virus for Transduction

1. Collect media 48 h after transfection, for PLAT-E cells also collect at 72 h (days 12, 13) into a 50 mL conical tube (the three pMX virus-containing media can be mixed together at this point).
2. Centrifuge at $3,000\times g$ for 5 min at room temperature to remove cells in the supernatant.
3. Filter supernatants using a $0.45\ \mu\text{m}$ pore-size polyethersulfone syringe filter to remove remaining cells.
4. Add polybrene to a final concentration of $4\ \mu\text{g/mL}$ to virus-containing media.
5. Use immediately for fibroblast transduction. The supernatant is approximately 30 mL when combined from all PLAT-E plates. The 30 mL is typically enough to transduce three 10 cm plate of fibroblasts with 10 mL of virus-containing media each.

3.3. iPSC Induction: Fibroblast Passaging and Transduction

The field of iPSC induction has changed rapidly since the initial paper by the Yamanaka group in 2006 (8) and new approaches that increase the efficiency of iPSC induction have been described (9–15). The human iPSC lines established in our laboratory so far were produced following the method developed in Shinya Yamanaka laboratory (except that we omit one of the factors, *c-MYC*) with modifications described by Sheng Ding and collaborators (1, 4, 9, 16). Here, we describe methods for iPSC induction in the presence (Sect. 3.3.1) and absence of feeder cells (3.3.2). We provide an overall timeline for the entire process of reprogramming, including preparation of virus for transduction, in Fig. 3. This timeline uses the same scheduling days across the procedure for experimental planning purposes.

3.3.1. iPSC Induction on SNL Feeder Cells

3.3.1.1. Procedure

1. Plate 5×10^5 fibroblasts 1 day prior to transduction with lentivirus (day 3) onto a 10-cm dish in complete DMEM media.
2. The following day (day 4), aspirate media and replace with prepared lentivirus-containing 293FT supernatant DMEM complete media *without* G418.

3. Incubate for 24 h and replace virus-containing media with fresh complete DMEM media (day 5).
4. Maintain cells for 7 days following transduction in complete DMEM media. Change media every 3 days. Be sure that the culture remains between 20 and 80% confluency throughout this time. If the culture reaches 80% confluency prior to day 11, passage with trypsin and replate cells at a 1:2 density (Sect. 3.1.2).
5. Passage 5×10^5 fibroblasts 1 day prior to transduction with retrovirus (day 11) onto a 10-cm dish in complete DMEM media.
6. The following day (day 12), aspirate media and replace with prepared retrovirus-containing PLAT-E supernatant complete DMEM media.
7. 24 h after first transduction replace virus-containing media with a second round of retrovirus-containing PLAT-E supernatant complete DMEM media (day 12).
8. Replace virus-containing media with complete DMEM media 24 h after second retroviral transduction (day 13).
9. Maintain cells for 7 days following initial retroviral transduction in complete DMEM media, changing the media every 3 days. Be sure that the culture remains between 20 and 80% confluency throughout this time. If the culture reaches 80% confluency prior to day 19, passage with trypsin and replate cells at a 1:2 density (Sect. 3.1.2).
10. One day prior to passing transduced fibroblasts (day 18), prepare a 6-well plate with SNL feeder cells (Sect. 3.5.3).
11. The following day (day 19), passage and plate 1×10^5 transduced fibroblasts into each well of the SNL feeder-coated 6-well plate in complete DMEM media.
12. The following day (day 20), replace complete DMEM media with hES media.
13. Continue to replace media every other day until the culture begins to deplete the media (color of media turns yellow), then feed daily.

A timeline for iPSC induction as outlined above is shown in Fig. 3.

3.3.2. iPSC Induction Under Feeder-Free Conditions

3.3.2.1. Procedure

1. Follow steps 1–9 described in Sect. 3.3.1.
2. One hour prior to passing transduced fibroblasts (day 19), prepare a Matrigel-coated 6-well plate.
3. Passage and plate 1×10^4 transduced fibroblasts into each well of the Matrigel-coated 6-well plate in complete DMEM media.

4. The following day (day 20), feed the cells with reprogramming media (hES media containing leukemia inhibitory factor (LIF) (10 ng/mL), PD0325901 (0.5 μ M), SB431542 (2 μ M), and Thiazovivin (0.5 mM).
5. Replace reprogramming media every other day until the culture begins to deplete the media (color of media turns yellow), then feed daily.

3.4. iPSC Colony Selection

After the viral transduction, the fibroblast cultures appear heterogeneous, composed of cells and cell colonies with different morphologies. The cells continuously change characteristics during the approximate 30-day period leading up to the formation of true iPSC colonies. 3-Factor induction (*OCT3/4*, *SOX2*, and *KLF4*) may prolong the time to the formation of human iPSCs when compared to inductions with 4 factors; however, it appears that 3-factor inductions reduce the number of partially induced iPSC colonies (4). Once presumptive colonies are identified, by light microscopy or live-immunocytochemical staining, the colonies are “picked” individually, and each is placed into a separate well of a 24-well plate and monitored by phase-contrast light microscopy. The recent development of live-cell immunocytochemistry with fluorophore-conjugated antibodies directed against stem cell surface markers has greatly increased the efficiency of selecting true iPSC colonies (17).

3.4.1. Colony Morphology Descriptions and Images

The progression of reprogramming can be monitored by morphological changes. Because cell doubling rates directly affect the rate at which somatic cells reprogram into iPSCs (18), differences in doubling rates of initial fibroblasts and their underlying epigenetic state can alter the timing of iPSC generation.

Most of the early forming colonies are likely to be non-iPSC colonies. These cells form circular patterns of distinct cells with spherical cell bodies that are often either phase bright or of a grainy darker appearance (Fig. 4a–c). These non-iPSC colonies often appear granular (phase darker) with cells piling up and have fuzzy colony margins rather than the smooth margin and flatter appearance observed with true iPSC colonies. Another class of non-iPSC colony morphology has more elongated cells that resemble small fibroblasts. These colonies vary in density and the densest colonies are easily misidentified as true iPSC colonies. True human iPSC colonies become uniformly circular as they expand (Fig. 4d), but when they are constrained by neighboring cells they can form elongated or tear-drop shaped colonies (Fig. 4e). The colony margins are defined and smooth compared to the “fuzzy” appearance of most non-iPSC colonies.

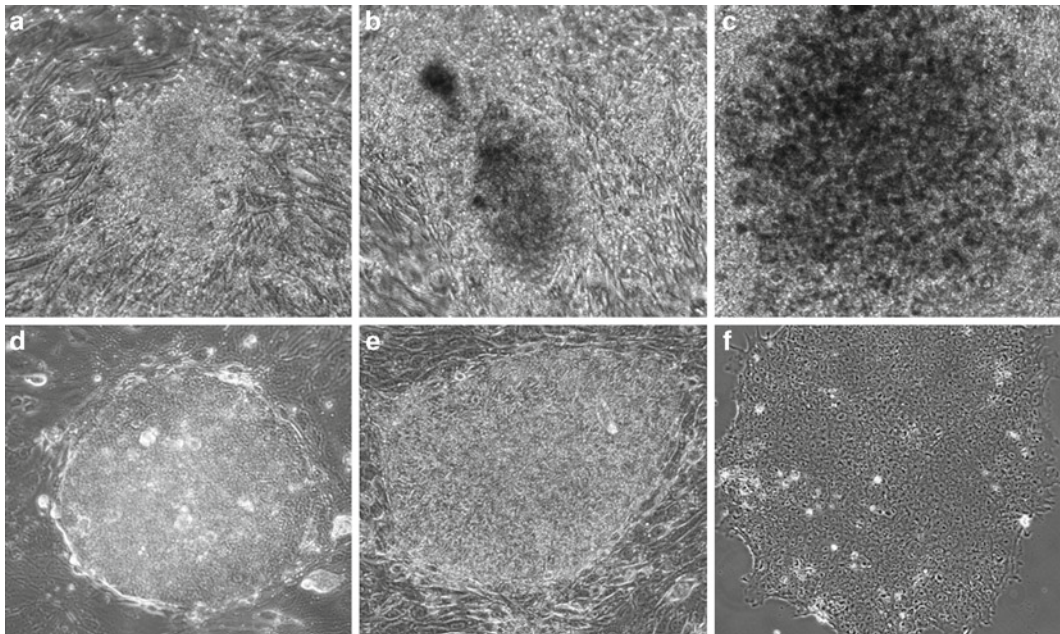


Fig. 4. Examples of various colony morphology types observed during reprogramming and propagation of iPSC colonies on feeders and Matrigel. (a–c) Partially reprogrammed human iPSC colonies form circular associations of cells appearing either phase bright (a) or phase dark (b, c). They usually do not show clearly defined colony margins and show an overall fuzzy appearance. The morphologies of these colonies are in sharp contrast to the smooth colony margins and flatter appearance of true iPSC colonies. (d, e) Human iPSCs derived from the fibroblasts of a normal subject are growing on feeder cells. These images display one human iPSC colony with surrounding SNL feeder cells. Human iPSC colonies can have a circular or oval/tear-drop shape. (f) The same human iPSC line can be grown under feeder-free conditions. One human iPSC colony growing on a Matrigel substrate is shown. The high nuclear to cytoplasmic ratio and prominent nucleoli typical for iPSCs is more easily appreciated in colonies growing on Matrigel than on feeder cells. All panels were imaged on a Zeiss microscope using a 10× phase contrast objective.

Human iPSCs have large round nuclei with prominent nucleoli and display a large nuclear to cytoplasmic ratio with the cytoplasm appearing phase darker than the nucleus. The overall cell shape is typically trapezoidal, but cell size and shape varies with colony size. These features are demonstrated in the higher magnification images of an iPSC colony grown on Matrigel (without feeders) shown in Fig. 7a. As a colony becomes larger, the cells are more tightly packed causing the cells to appear smaller. Cells in small colonies or at the margins of colonies growing in feeder-free conditions appear larger than the cells in the colony center. Borders between iPS cells often appear phase bright under a light microscope.

3.4.2. Live Imaging of Human iPSC Markers

For live imaging, cells are incubated with fluorophore-conjugated antibodies against iPSC markers (Fig. 5). Live staining can therefore only be performed with antibodies directed against cell surface-associated antigens. We typically use fluorophore-conjugated antibodies against SSEA4, TRA-1-60, and TRA-1-81 (for source

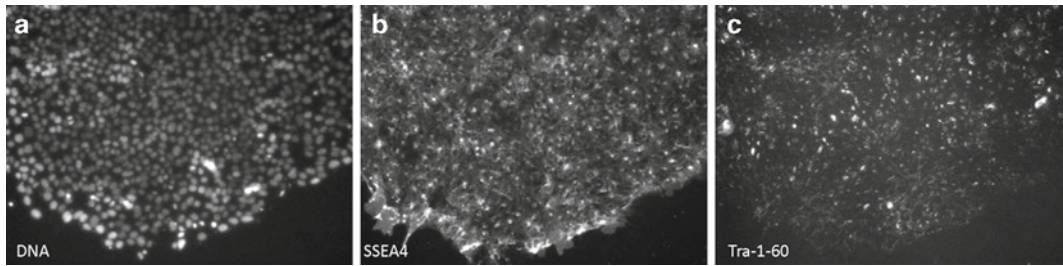


Fig. 5. Live staining of presumptive human iPSC colonies. This human iPSC colony was triple stained with Hoechst (DNA) to visualize all cell nuclei and the two iPSC surface markers SSEA4 and TRA-1-60.

and dilution of antibodies see Table 1). Live staining permits the early identification of emerging reprogrammed cells and therefore is a useful technique for picking human iPSC colonies, but is also helpful for monitoring the status of established iPSC lines and their differentiation. The following protocol was adapted from a published method (17).

3.4.2.1. Procedure

1. Dilute TRA-1-60-Alexa Fluor 555 and SSEA4-Alexa Fluor 488 (see Table 1) in warm mTeSR1 media.
2. Gently aspirate media from cultures to be stained.
3. Add antibody-containing media to cells (0.25 mL/well or 0.75 mL/well for 24- or 6-well plates, respectively).
4. Incubate for 1.5 h at 37°C.
5. Add Hoechst dye to wells (0.5 µg/mL final concentration).
6. Incubate for 30 min at 37°C.
7. Wash three times with DMEM/F12 media.
8. Image in hES media or mTeSR1 media.
9. Colonies can be directly picked under epifluorescence with the following protocol. Alternatively, the plate can be marked with a pen and the colony can be picked under phase-contrast light microscopy in a laminar flow hood to maintain sterility (see Note 2).

3.4.3. Picking Colonies onto SNL Cell Coated Culture Dishes

3.4.3.1. Procedure

1. Make a SNL cell 24-well feeder plate 1 day prior to picking colonies (Sect. 3.5.3).
2. Just prior to picking, wash the SNL plate twice with PBS and add 0.5 mL hES media with ROCK inhibitor, Y-27632 at a final concentration of 10 µM.
3. Identify colonies by morphology or live cell imaging (Sect. 3.4.2).
4. Pick colonies using a pulled-glass scraper (Fig. 6a). Since each colony represents one separate hiPSC cell line, it is important that colonies are kept separate by ensuring that they are

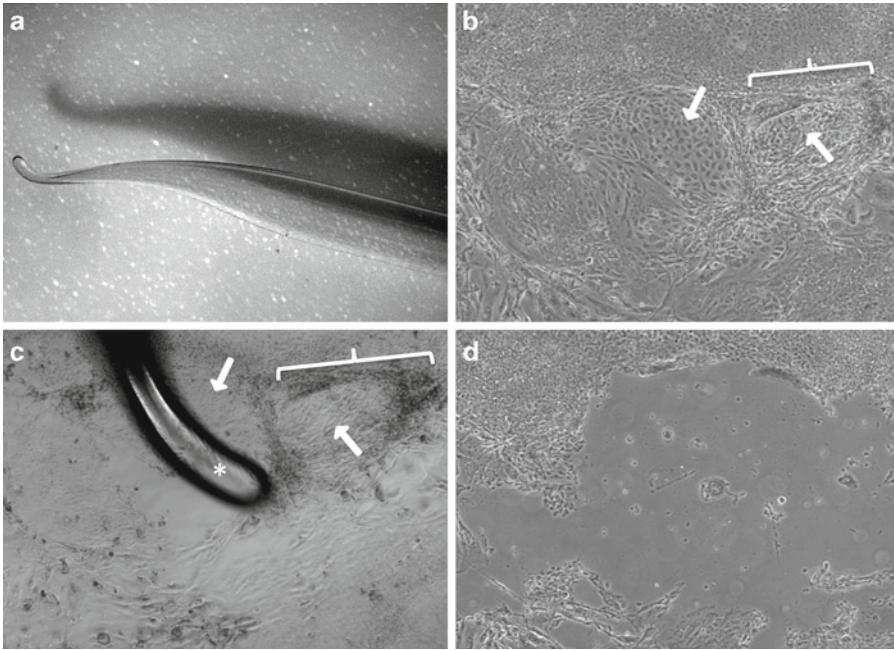


Fig. 6. Removal of differentiated cells from iPSC colonies. During propagation some spontaneous differentiation of a subset of human iPSCs is to be expected. To maintain the purity of the culture, these cells must be removed before passaging. **(a)** We prepare a scraping tool from Pasteur pipettes for that purpose. **(b)** We routinely observe two types of differentiation, cells that appear more spread out and are somewhat phase darker than human iPSCs (*arrows*). These cells are usually observed in the center or the edge of a colony. The other types of changes resemble more a piling up of human iPSCs often in the center or the edge of the colony (*area under bracket*). **(c)** These cells are scraped from the dish surface using the glass scraper (*star*) and then aspirated. The areas of differentiation from **(b)** are highlighted again (*with arrows and under the bracket*). **(d)** Area of differentiation has been removed with the scraping tool. The remaining human iPSCs are then passaged. **(b–d)** Imaged on a Zeiss microscope using a 10× phase contrast objective.

individually picked and transferred to the new culture dish. With a glass scraper gently separate the colonies one at a time from the SNL feeder cells.

5. Transfer the colony or colony fragments by pipetting them slowly into a 1 mL glass serological pipette and then place each colony (or the fragments of one colony) into a separate well of a SNL-coated 24-well plate.
6. Once colonies have grown substantially, the hiPSCs are passaged using the CTK method (see Sect. 3.5.3) and plated into 1 well of a 12-well SNL cell-coated culture dish.
7. For the next passage, plate the cells into 1 well of a SNL cell-coated 6-well dish.
8. Then continue to expand the hiPSC lines for verification, cryopreservation, and experiments as needed (a minority of original colonies picked will actually expand into iPSC lines).

3.4.4. Picking Colonies onto Matrigel-Coated Culture Dishes

1. Coat a 24-well tissue culture plate with Matrigel following the vendors' protocol.
2. Add fresh reprogramming media (hES media containing LIF (10 ng/mL), PD0325901 (0.5 μ M), SB431542 (2 μ M) and thiazovivn (0.5 μ M)) to the cultures from which colonies will be picked.
3. Remove Matrigel solution and add 0.5 mL of reprogramming medium containing 10 μ M ROCK inhibitor to each well.
4. Since each colony represents one separate hiPSC cell line it is important that colonies are kept separate by ensuring that they are individually picked and transferred to the new culture dish. With a pulled-glass scraper (Fig. 6a) gently remove the fibroblasts surrounding the hiPSC colony and carefully separate the colony from the Matrigel-coated dish surface. If the colony is large, break it into several pieces; small colonies should be kept intact if at all possible.
5. Transfer the colony fragments by pipetting them slowly into a 1 mL glass serological pipette and placing each colony (or the fragments of one colony) into a separate well of the Matrigel-coated 24-well plate.
6. One day after picking, feed the cells with reprogramming media (without ROCK-inhibitor).
7. Next day feed the cells with media containing 75% reprogramming media + 25% mTeSR1 media (note that the concentration of LIF, PD0325901, Thiazovivin, and SB431542 will be reduced with increasing increments of mTeSR1 medium).
8. Next day change to 50%/50% reprogramming/mTeSR1 media.
9. Next day change to 25%/75% reprogramming/mTeSR1 media.
10. Next day change to 100% mTeSR1.
11. Once colonies have grown substantially, scrape away any non-iPSC-like cells with a glass scraper, and passage the hiPSCs using dispase (see Sect. 3.5.1) and plate them into 1 well of a 12-well Matrigel-coated culture dish.
12. When passaging the cells again plate them into 1 well of a Matrigel-coated 6-well dish.
13. Then continue to passage and expand the hiPSC lines for verification, cryopreservation, and experiments as needed (a minority of original colonies picked will actually expand into iPSC lines).

3.5. Maintenance and Expansion of iPSC Colonies

Once primary iPSC colonies have been picked, they have to be expanded for long-term storage and validation (for validation of human iPSCs, see Sect. 3.6). Human iPSCs require a combination of different factors for their survival and proliferation.

Traditionally, these factors are provided by co-cultured feeder cells or media conditioned by mouse embryonic fibroblasts (MEF) (2, 3). We use mitomycin-C inactivated SNL cells (1, 7), which are a derivative of the STO mouse fibroblast cell line which secretes murine LIF and is transformed with neomycin resistance (19). Irradiated MEF have also been used as feeder cells and may be substituted for the SNL cells described here (2, 3). Examples of typical human iPSC colonies growing on SNL feeder cells are portrayed in Fig. 4d, e. As observed previously (16), we noticed that the time point and plating density of SNL cells can greatly affect iPSCs growth. Thus, care should be taken that SNL cells are plated at the appropriate density 1–2 days before the passage of the iPSCs.

More recently, human stem cells have also been successfully cultured in the absence of feeder cells (20–22). Growing iPSCs in the absence of feeder cells has several advantages which include the possibility to use defined media, unobstructed visualization of human iPSC colonies, elimination of the need to maintain and prepare an additional cell type (feeder cells), avoidance of potentially confounding feeder cells during iPSC differentiation, and better recovery of frozen iPSCs. We describe methods for culturing human iPSCs on SNL feeder cells as well as under feeder-free conditions on dishes coated with Matrigel, a commercially available mixture of extracellular matrix proteins. However, for the reasons mentioned above, we now almost exclusively culture human iPSCs on Matrigel. A human iPSC colony growing on Matrigel is shown in Fig. 4f.

Freezing and reconstituting iPSCs can be a challenge and various methods for freezing and thawing stem cells have been described (16, 20, 23). In our hands, a rapid freeze/rapid thaw procedure proved best for human iPSCs that are to be plated onto SNL feeder cells. However, for plating human iPSCs onto Matrigel a slow freeze/rapid thaw approach was more successful.

All our protocols have been adapted to human iPSC cells grown in standard 6-well tissue culture plates; and thus, all volumes indicated need to be adjusted if other culture dish sizes are used.

3.5.1. Passaging Human iPSCs on Matrigel

To culture human iPSCs under feeder-free conditions, the cells are seeded and maintained in culture dishes coated with Matrigel. Matrigel is a soluble basement membrane extract mainly composed of laminin, collagen IV, entactin, and heparan sulfate proteoglycan (24, 25). Human iPSCs growing on Matrigel are maintained in a defined serum-free media (mTeSR1) that is changed daily. Once seeded onto Matrigel, the colonies expand and need to be passaged about every 4–5 days or when about 80% of the culture surface is covered with colonies.

As human iPSCs are maintained in culture, a subset of the cells spontaneously differentiate. The two most frequent types of differentiation we observe are shown in Fig. 6b. These differentiated cells need to be removed before each passage. We remove these cells scraping them off the dish surface with glass scrapers that we prepare by pulling glass Pasteur pipettes in a flame (Fig. 6a). To maintain sterility of the iPSC cultures, we move an inverted light microscope into the tissue culture hood. If the media is changed daily and the cells are passaged at appropriate intervals (4–5 days), the percentage of cells that differentiate should not exceed 5–10%. Extending the time between passaging or omitting daily media changes results in increased differentiation of the cells. In addition, we found that the degree of differentiation varies among different human iPSC lines, even among lines derived from the same fibroblasts.

3.5.1.1. Procedure

Although widely performed, we found that a preincubation with ROCK inhibitor before enzymatic treatment of human iPSCs is not necessary.

Preparation for Passaging

1. At least 1 h before cell passaging, the culture dishes to be used are coated with Matrigel using the protocols provided by the manufacturer.
2. The differentiated cells characterized by the morphologies described above are removed with a glass scraper prepared from Pasteur pipettes (Fig. 6) with the aid of an inverted light microscope that is moved into the tissue culture hood.

Enzyme Treatment

1. Aspirate media including the scraped differentiated iPSCs from the cultures.
2. Wash cultures once with DMEM/F12 media.
3. Add 1 mL/well of dispase and incubate for 8–13 min depending on the lot of enzyme. Start observing the cultures after about 8 min; the edges of colonies first will become phase bright and then start to curl up (Fig. 7b). When the edges along the full perimeter of the colonies just begin curling up (Fig. 7c), stop the digestion by aspirating the dispase solution (Note 4). Do not allow the dispase to act past the degree of curling shown in Fig. 7d because that will result in loss of cells.
4. Gently wash the cells three times with 2.5 mL DMEM/F12 media.

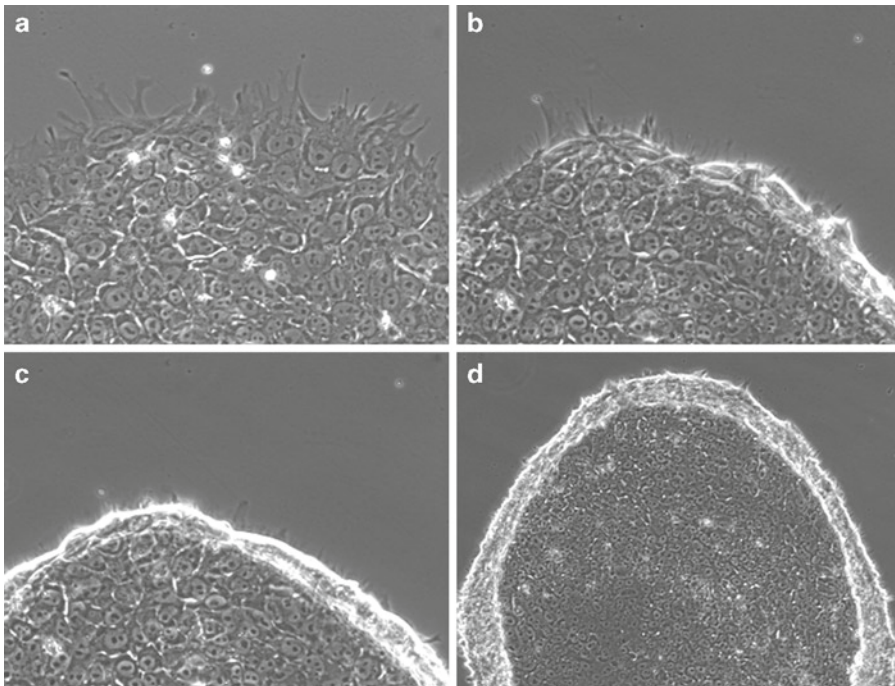


Fig. 7. Treatment of human iPSC colonies with dispase. (a) Human iPSC colonies growing on Matrigel have a very flat appearance with the edges of the colonies tightly adhering to the Matrigel covered dish surface. Of note, the high nuclear to cytoplasmic ratio and the prominent nucleoli typical for iPSCs are easily appreciated in these close-up images. (b) Exposure to dispase for ~11 min results in the curling of the edges of the colonies. (c) Two minutes later (~13 min) is the time when dispase treatment should be stopped and colonies harvested. Images (a–c) were obtained using a 20× objective. (d) An image of the same iPSC colony obtained with a 10× objective provides a view of a larger area of the dispase treated colony. Do not allow the dispase treatment to continue past this stage; otherwise you risk losing the colonies.

Harvesting and Replating the Cells

1. Add 2 mL/well of mTeSR1 media containing 10 μ m ROCK inhibitor Y-27632, a compound that promotes the survival of dissociated human stem cells (26).
2. Scrape colonies with a cell scraper (see Note 5).
3. Transfer the cell suspension into a 50 mL conical tube.
4. Add an additional 2 mL of mTeSR1 media containing 10 μ m ROCK inhibitor into each well to remove remaining cells.
5. Transfer cell suspension into same 50 mL conical tube.
6. Add an additional 6 mL of mTeSR1 media containing 10 μ m ROCK inhibitor to the 50 mL conical tube. This procedure dilutes iPSCs by a factor of 5; depending on the desired dilution of the iPSCs, volume of media added can be modified. We found splitting ratios between 1:3 and 1:5 to be optimal.
7. Further break up colonies by gently pipetting the cell suspension up and down three to five times with a 5 mL glass serological pipette (see Note 6).

8. Transfer cells onto Matrigel-coated dishes (2 mL/well) using a glass serological pipette. If a transfer of human iPSCs from Matrigel to feeder cells is desired, the human iPSCs can at this stage be plated onto prepared feeder cells instead of Matrigel (see also Sect. 3.5.3).
9. Put plates in incubator and move them back and forth and left to right a few times (this will result in an even distribution of human iPSC clumps over the whole surface).
10. Feed and observe daily for growth of colonies.

3.5.2. Freezing and Thawing of Human iPSCs for Propagation on Matrigel

3.5.2.1. Procedure

Human iPSCs should be frozen at the time when they are ready for passaging or the day before. We have obtained optimal recovery of frozen cells by freezing cells harvested from 1 well of a 6-well plate in 1 mL of freezing solution and replating these cells back into 1 well of a 6-well plate.

Freezing Human iPSCs

To prepare human iPSCs for freezing, follow steps 2–5 described previously (Sect. 3.5.1) with the following modifications:

- (a) ROCK inhibitor does not need to be included in the mTeSR1 media for the collection of the cells from the dishes.
- (b) Cells from each well are collected in a separate 15 mL conical tube (cells collected from each well will be frozen in a separate cryo-vial).

then continue as outlined:

1. Spin cell suspensions at $120\times g$ for 5 min at room temperature.
2. Aspirate media.
3. Add 1 mL feeder-free iPSC freezing solution to the cell pellet and transfer the resuspended cells into a cryo-vial using a 1 mL glass serological pipette (pipetting while resuspending the cell pellet should be kept to an absolute minimum).
4. Put cryo-vials into Nalgene freezing container for overnight freezing at -80°C .
5. Transfer the cells into liquid nitrogen storage facility the next day.

Thawing and Plating of Human iPSCs

1. Coat culture dish with Matrigel at least 1 h before thawing the cells.
2. Rapidly transfer the cryo-vial containing the human iPSCs from the liquid nitrogen storage into a 37°C water bath.
3. Thaw the cells by gently agitating until most but not all of the content is thawed.

4. Remove the vial from the water bath and let the remainder of the cells thaw.
5. Rinse the vial with 75% ethanol before transferring it into the tissue culture hood.
6. Transfer the thawed cell suspension into a 15 mL conical tube using a 1 mL glass serological pipette.
7. Slowly add 9 mL prewarmed mTeSR1 media to the cells.
8. Spin cells at $120 \times g$ for 5 min at room temperature.
9. Aspirate media and resuspend the cell pellet in 2.5 mL of mTeSR1 media containing 10 μ M ROCK inhibitor.
10. Plate the cells into 1 well of a 6-well culture dish coated with Matrigel.

3.5.3. Passaging Human iPSCs onto SNL Feeder Cells

Commonly iPSCs have been grown on feeder cells to maintain pluripotency. We use SNL feeder cells that are propagated and mitomycin-C treated according to published protocols (16). We passage human iPSC cells growing on SNL cells about once every 5 days. Shorter and longer intervals between passaging result in lower yields and increased human iPSC differentiation, respectively. Immediately before passaging, differentiated human iPSCs identified by the morphologies described previously (Fig. 6) are removed by scraping. To passage human iPSCs on SNL cells, we use a protocol similar to the procedure described by Yamanaka and colleagues (16).

3.5.3.1. Procedure

Preparation for Passaging

1. The day before passaging, SNL cells are seeded into gelatin-coated dishes at a density of 2.5×10^4 cells/cm² in complete DMEM media.
2. Shortly before passaging, the differentiated human iPSCs are removed with a glass scraper as described previously (Sect. 3.5.1).
3. On the day the iPSCs are passaged, the SNL cells are washed two times with D-PBS and then transferred into hES media until plating of the human iPSCs.

Enzyme Treatment

1. Aspirate media from the cultures.
2. Wash cells with 2.5 mL D-PBS once.
3. Aspirate D-PBS completely.
4. Incubate cells in 400 μ L/well of CTK solution for 3–5 min in incubator (start observing detachment of SNL cells at 3 min, too long of an enzyme exposure will result in the loss of human iPSC colonies).

5. When 90% of SNL cells are detached, add 2.5 mL of D-PBS to the wells.
6. Aspirate CTK/D-PBS solution and repeat washing step once.
7. Remove D-PBS completely.

Harvesting and Replating the Cells

1. Add 2 mL of hES media to each well.
2. Scrape human iPSC colonies using a cell scraper.
3. Transfer cells into 50 mL conical tube.
4. Rinse each well with 2 mL of hES media and add to the 50 mL conical tube.
5. Repeat step 4.
6. Break the colonies into smaller clumps by pipetting up and down (three to five times) with 5 mL glass serological pipette.
7. Replate cells onto mitomycin-C treated SNL cells (see Note 7).
8. Put plates in the incubator and move a couple of times back and forth and left and right to distribute cells evenly on the dish surface.
9. Feed and observe daily.

3.5.4. Freezing and Thawing of Human iPSCs for Propagation on SNL Feeder Cells

For freezing and thawing human iPSCs to be plated onto SNL feeder cells, we follow the procedures described by Ohnuki et al. (16).

3.6. Validation of Human iPSCs

Once human iPSC lines are established, their pluripotency status has to be validated. This is achieved by evaluating a set of criteria that has been established by multiple groups. Typically, the first tests to be performed are analysis of cell and colony morphology and the presence of alkaline phosphatase activity. These tests are relatively cheap, easy, and quickly performed and thus serve as an early screen for potential human iPSC clones. Karyotyping of the human iPSCs is typically performed after human iPSCs have been propagated to demonstrate that these cells maintain a stable karyotype that is equivalent to the fibroblast line they have been derived from. Expression of pluripotency markers is assessed by immunocytochemistry and RT-PCR. Bisulfite genomic sequencing is used to assess the methylation status in the promoter regions of pluripotency-associated genes. Finally, pluripotency must be demonstrated by the potential of the human iPSCs to differentiate in vitro and/or in vivo into cell types derived from the three embryonic germ layers: ectoderm, mesoderm, and endoderm.

3.6.1. Assessment of Colony and Cell Morphology

Colony and cellular morphological characteristics are assessed by phase/contrast microscopy. Typical human iPSC colonies are formed from tightly packed cells, are of round or oval shape with well-defined edges. The cells within these colonies show a high nuclear to cytoplasmic ratio and prominent nucleoli (Figs. 4d–f and 7a).

3.6.2. Assessment of Alkaline Phosphatase Activity

Alkaline phosphatase activity has been proposed to be the most reliable pluripotency marker for human stem cells (27). In order to assess the pluripotency of potential human iPSCs, the colonies need to be propagated in duplicate, one culture is used for alkaline phosphatase activity (fixation is needed for this step and thus this procedure terminates the culture), the sister culture is used for propagation if alkaline phosphatase staining confirms the potential iPSC character of the line. We test for alkaline phosphatase enzymatic activity of the human iPSCs using an alkaline phosphatase staining kit available from Sigma exactly following the manufacturer's recommendations.

3.6.3. Immunocytochemical Assessment of Pluripotency Markers

Pluripotency markers identified in hES cells are also markers of human iPSCs and include SSEA3, SSEA4, TRA-1-60, and TRA-1-81 surface antigens and transcription factors such as OCT4, NANOG, and SOX2. An example of immunofluorescence staining of a human iPSC line made in our lab for the markers OCT4, SSEA3, SSEA4, and TRA-1-60 is shown in Fig. 8. Staining for surface markers has the advantage that live staining can be performed (see Sect. 3.4.2). For the staining of intracellular

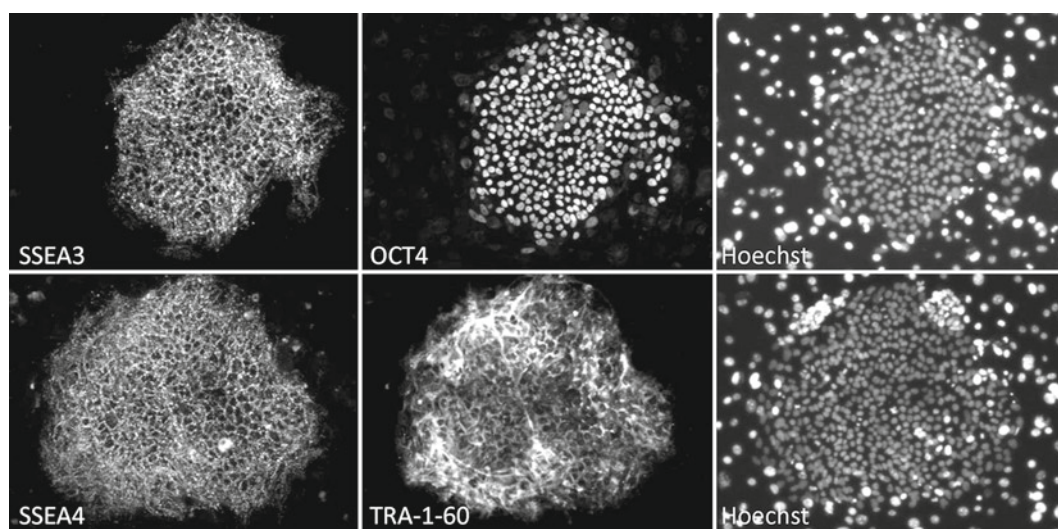


Fig. 8. Validation of human iPSC lines by immunocytochemistry. These human iPSCs cells (BG8 line) derived from a skin biopsy of a normal subject were assessed for the expression of human iPSC marker proteins. Here we show one colony that was stained for the surface marker SSEA3 and the transcription factor OCT4. Another colony of the same cell line demonstrates co-expression of the surface markers SSEA4 and TRA-1-60. The cultures were counter stained with the Hoechst dye to visualize all cell nuclei. Intensely stained feeder cell (SNL) nuclei are seen outside the perimeter of the human iPSC colonies.

proteins, the cells have to be fixed, a procedure that terminates the cultures. In order to better evaluate the results obtained from a new human iPSC line under investigation, it is critical to include an already established human iPSC or hES cell line in the staining procedure as a positive control. Counterstaining with a Hoechst (DNA) dye that labels the nuclei of all cells allows the investigator to determine the percentage of cells that expresses a particular marker of interest (Fig. 8). Since these methods are not able to accurately measure relative expression levels, assessment of gene expression by quantitative RT-PCR (see Sect. 3.6.4) is often used as a companion validation approach.

3.6.3.1. Procedure

1. Aspirate media.
2. Rinse cells once with D-PBS.
3. Fix cells with 4% paraformaldehyde in PBS for 30 min at room temperature.
4. Wash cells 3× 5 min with PBS.
5. Permeabilize cells with 0.2% Triton X-100 in PBS for 20 min at room temperature.
6. Incubate cells in PBS⁺⁺ (PBS + 5% donkey serum + 0.05% TX-100) for 2 h at room temperature or overnight at 4°C.
7. Incubate cells with primary antibodies (see Table 1) diluted in PBS⁺⁺ overnight at 4°C.
8. Wash cells 5× 10 min with PBS⁺ (PBS + 0.05% Triton X-100).
9. Incubate cells with secondary antibodies (see Table 1) diluted in PBS⁺⁺ for 3 h at room temperature or overnight at 4°C.
10. Wash cells 5× 10 min with PBS⁺ at room temperature.
11. Wash 1× 5 min in PBS at room temperature.
12. Stain with Hoechst dye at 2 µg/mL for 5 min in PBS.
13. Wash 3× 5 min in PBS.
14. Image with a light microscope equipped for epifluorescence.

3.6.4. Additional Assays Used for the Validation of Human iPSCs

In addition to the above described assays, other criteria are used to characterize and validate human iPSC lines, including evaluation of expression levels of iPSC markers, assessment of gene activation, chromosomal stability, and potential of the cells to differentiate (see Sect. 3.6.5). Activation of the endogenous alleles of the pluripotency inducing factors (i.e., *c-MYC*, *SOX2*, *KLF4*, *OCT4*) as well as other key markers of pluripotency (e.g., *NANOG*) coupled with downregulation of the viral expressed factors are critical steps in generating iPSCs (1–3, 17, 28). Quantitative RT-PCR is a valuable tool to assess expression of stem cell markers. With this assay, relative expression levels can be assessed over time or between different iPSC lines for activation of endogenous genes, as well as monitoring expression of the viral transgenes. As these assays are

relatively straight forward, detailed methods are not provided here. The assay begins with the extraction of RNA from the iPSCs, which is then used as a template by a reverse-transcriptase reaction to generate cDNA. The cDNA of interest, determined by the primers chosen, is quantified by PCR amplification and product levels monitored as a function of real time to provide a measure of the initial concentration of RNA (29, 30). There are numerous commercially available kits for RNA purification, primer design, cDNA generation, and RT-PCR that have been successfully used. Table 3 provides a list of primers that we have successfully used in

Table 3
Validated human iPSC primers for SYBR green real-time RT-PCR

Primer	Tm	Sequence
<i>GDF3</i> Forward	60.0	AAATGTTTGTGTTGCGGTCA
<i>GDF3</i> Reverse	60.0	TCTGGCACAGGTGTCTTCAG
<i>ACTB</i> Forward	60.4	TGAAGTGTGACGTGGACATC
<i>ACTB</i> Reverse	58.4	GGAGGAGCAATGATCTTGAT
<i>NANOG</i> Forward	58.4	TGAACCTCAGCTACAAACAG
<i>NANOG</i> Reverse	58.4	TGGTGGTAGGAAGAGTAAAG
<i>hTERT</i> Forward	58.4	TGTGCACCAACATCTACAAG
<i>hTERT</i> Reverse	60.4	GCGTTCCTTGGCTTTCAGGAT
<i>OCT4</i> endogenous Forward	60.4	CCTCACTTCACTGCACTGTA
<i>OCT4</i> endogenous Reverse	58.4	CAGGTTTTCTTTCCCTAGCT
<i>OCT4</i> total Forward	60.4	AGCGAACCAGTATCGAGAAC
<i>OCT4</i> total Reverse	60.4	TTACAGAACCACACTCGGAC
<i>SOX2</i> endogenous Forward	60.2	CCCAGCAGACTTCACATGT
<i>SOX2</i> endogenous Reverse	60.4	CCTCCCATTTCCTCGTTTT
<i>SOX2</i> total Forward	60.4	AGCTACAGCATGATGCAGGA
<i>SOX2</i> total Reverse	60.4	GGTCATGGAGTTGTACTGCA
<i>KLF4</i> endogenous Forward	60.4	GATGAACTGACCAGGCACTA
<i>KLF4</i> endogenous Reverse	60.4	GTGGGTCATATCCACTGTCT
<i>KLF4</i> total Forward	62.4	TCTCAAGGCACACCTGCGAA
<i>KLF4</i> total Reverse	60.4	TAGTGCCTGGTCAGTTCATC
<i>DNMT3B</i> Forward	60.4	ATAAGTCGAAGGTGCGTCGT
<i>DNMT3B</i> Reverse	58.4	GGCAACATCTGAAGCCATTT

Primer sequences match those published by Chan et al. (17).

our lab for validation of human iPSCs with SYBR green real-time RT-PCR. Sequences of additional primers used for the assessment of human iPSC lines can be obtained from published protocols (7, 16, 17, 31). The reader is encouraged to consult these publications for information on the details of these assays.

Gene activation is determined in part by the epigenetic status of promoter DNA methylation. Methylated CpG islands are usually clustered around regulatory regions of genes and are associated with gene inactivation. Thus, the determination of the methylation status of promoters flanking pluripotency markers is another useful tool to evaluate human iPSCs, where the promoter regions of *OCT4*, *NANOG*, and other pluripotency markers should be hypomethylated relative to the DNA methylation status of the parent fibroblast cells. The methylation status of genomic DNA can be determined by bisulfite sequencing. In this procedure, bisulfite treatment of genomic DNA is performed using commercially available kits (for example, the EpiTect Bisulfite Kit by Qiagen) according to the manufacturer's recommendations. The modified DNA is then amplified by PCR using primers within the promoters of interest. The amplified sequences are cloned and randomly selected clones sequenced. For procedures and PCR primer information, the reader is referred to published methods (6, 8).

Cell propagation can result in chromosomal instability. Thus, karyotyping is performed to ensure that propagated human iPSCs maintain a stable karyotype that is equivalent to the one of the fibroblasts they were derived from. Different methods for karyotyping have been developed and the reader is referred to protocols used in the context of iPSCs (20, 22, 32).

3.6.5. Assessment of the Potential of Human iPSCs to Differentiate

If the presumptive iPSCs are indeed pluripotent stem cells, they should be capable of differentiation into cells derived from any of the three embryonic germ layers. The *in vivo* pluripotency of human iPSCs can be assessed by their potential to form teratomas in immunocompromised mice. Human iPSCs are injected into the mice and the resulting teratomas histologically assessed according to published protocols (6, 8, 22). True pluripotent human iPSCs should form teratomas comprised of tissues derived from all three embryonic germ layers. This technique, however, takes at least 8 weeks to perform and requires specialized care and evaluation of immunocompromised mice. Alternatives include *in vitro* differentiation to see if putative iPSC lines can form cells derived from all three embryonic germ layers. The protocols to induce differentiation *in vitro* into different cell types vary greatly depending on the desired cell type, and the reader is referred to the literature most appropriate to the cell type to be derived. The potential of human iPSCs to differentiate into any cell type of interest makes them a powerful tool for developing human models of toxicity. Here, we will describe a two-step method to derive neurons from human iPSCs. In the first step, human iPSCs are induced to

form neuronal progenitors, which are then further differentiated into neurons in the second step.

3.6.5.1. Derivation of Neuronal Progenitor Cells

Recently, methods have been published to perform neuronal inductions on a feeder-free system (5). Derivation of neurons from human iPSCs in the absence of feeder cells has several advantages, including higher yield of neurons, faster differentiation, and easier assessment of derived neurons. We perform all our neuronal differentiations with human iPSCs growing on Matrigel. SMAD signaling has been shown to play an important role during neural induction in several different systems (33–36). A recent study provides evidence that the combined use of Noggin, a bone morphogenic protein (BMP) inhibitor and SB431542, an inhibitor of the Lefty/Activin/TGF β pathways greatly increases the efficiency of neural induction (5).

To induce neuronal progenitor cells, human iPSC colonies are dissociated with Accutase, and the resulting single-cell suspension is plated onto Matrigel-coated 24-well plates. The density at which the human iPSCs are plated at this time affects their tendency to differentiate towards PAX6 positive CNS or PAX6 negative neural crest-like progenitors, with higher plating densities favoring differentiation towards PAX6 positive cells (5). Three to five days after plating, neuralization is initiated by incubating the cells in neuralization medium containing two different compounds that inhibit SMAD signaling (5). The potential neuroprogenitor cells are evaluated by their expression of neuronal progenitor cell markers (PAX6, NESTIN) and the absence of human iPSC markers (OCT4, TRA-160, TRA-1-81, SSEA4) using the immunocytochemical staining and quantitative RT-PCR described in Sects. 3.6.3 and 3.6.4, respectively (Fig. 9).

Procedure

Preparation

1. Prepare Matrigel-coated 24-well plates according to manufacturer's instructions.
2. Remove all differentiated colonies or parts of colonies from the culture.
3. Immediately before use thaw Accutase solution.

Neuralization of human iPSCs

1. Aspirate media.
2. Wash cells with D-PBS once (2.5 mL/well).
3. Aspirated D-PBS completely.
4. Incubate with 1 mL/10 cm² of culture surface (i.e., 1 mL/well of a 6-well plate) of Accutase solution in the incubator for no more than 8 min.

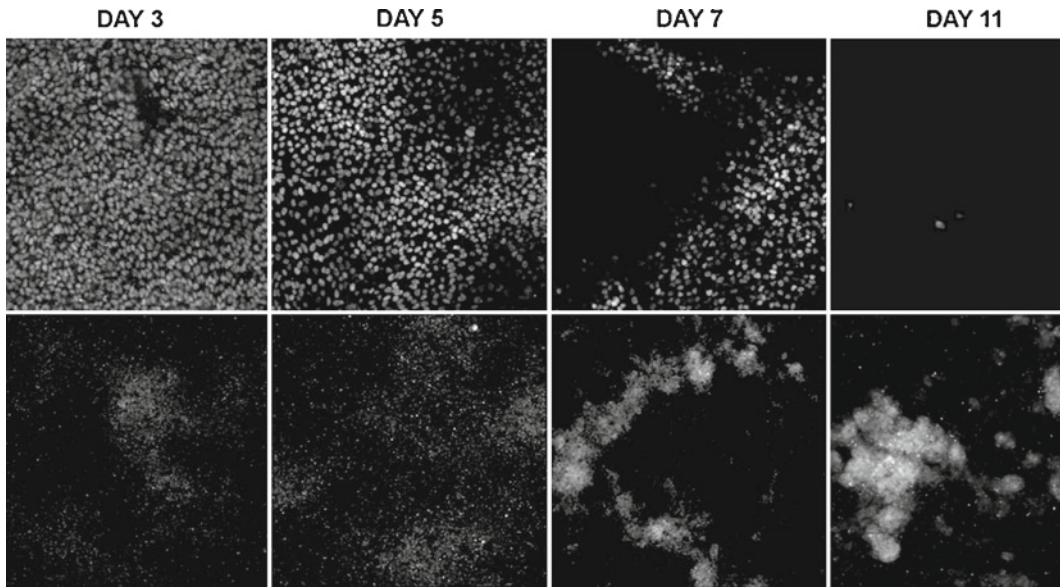


Fig. 9. Neural induction. Human iPSC line BG8 was exposed to noggin, a bone morphogenic protein (BMP) inhibitor and SB431542, a compound that inhibits the Lefty/Activin/TGF β pathways to induce the formation of neural progenitor cells. The expression of the iPSC marker OCT4 (*top*) and the neural progenitor marker PAX6 (*bottom*) as well as other markers of neuralization (data not shown) was examined by immunofluorescence staining. Over a 3–11 day period of neural induction, the level of OCT4 expression decreases while the signal of PAX6 increases.

5. Add 3 mL of mTeSR1 media containing 10 μ m ROCK inhibitor for every 1 mL of Accutase solution.
6. Pipette cells up and down several times to wash cells off the dish surface and to break up the remaining cell clumps into a single-cell suspension using 5 mL glass serological pipettes.
7. Transfer cell suspension into 15 mL conical tube.
8. Centrifuge cells 200 $\times g$ for 5 min at room temperature.
9. Aspirate medium and resuspend cells in 4 mL of mTeSR1 media containing 10 μ m ROCK inhibitor.
10. Centrifuge cells 200 $\times g$ for 5 min at room temperature.
11. Resuspend cells in 1 mL of mTeSR1 media containing 10 μ m ROCK inhibitor.
12. Count cells and dilute them to appropriate concentration: for the derivation of anterior CNS progenitor cells we plate at 18,000 cells/cm² as reported by others (5).
13. Plate cells onto Matrigel-coated dishes (we usually use 96-well plates for neuronal differentiations).
14. Feed and observe cells daily.
15. Within 3–5 days (depending on the human iPSC line) the cells are confluent and neuralization is initiated.

16. Cells are exposed to 10 μm SB431542 and 500 ng/mL Noggin in neuralization media for 5 days with a daily change of media.
17. Neuronal differentiation is initiated between day 5 and 11 and the protocols vary depending on the neuron type desired.

Once neuronal precursors have been derived and validated, they can be differentiated into different types of neurons. Depending on the neuronal type to be derived, the neuronal progenitor cells are exposed to varying series of combinations of factors for varying time periods. The description of all the different neuronal patterning protocols is beyond the scope of this chapter and the reader is referred to the diverse published methods. In principle, methods that have been developed for differentiation of hES cells are often transferrable to human iPSCs. For example, Studer and colleagues have reported the directed differentiation of both midbrain dopamine and spinal motor neurons from hES cells and human iPSCs using the same method (5). Indeed, these methods were originally developed with hES cells (37, 38). A selection of recently published papers and reviews with methods for generating a variety of neural cell types (e.g., neural progenitors, neural crest cells, peripheral sensory neurons, dopaminergic neurons, spinal motor neurons, serotonergic neurons, striatal DARPP32 positive neurons, inhibitory GABAergic, and excitatory glutamatergic neurons) is provided as a reference (37–47).

4. Notes

1. It is important to keep track of the number of passages performed with the fibroblasts. iPSCs appear to be more easily derived from early passage fibroblasts, thus cryopreservation of early passage fibroblasts is crucial.
2. The intensity of immunofluorescence in live cell imaging is much less than seen in typical immunocytochemistry. For this reason, positive and negative controls are vital for proper interpretation of live-cell fluorescence images. For optimal results, antibody concentrations should be titrated on positive control cells such as established human iPSC lines or hES lines.
3. Matrigel and SNL feeders along with the corresponding media (mTeSR1 and hES respectively) are both adequate methods for maintaining iPSC cells. However, Matrigel is preferable when growing to high density, live-cell imaging, or freezing.
4. It is important to closely watch the progress of the dispase digestion. As soon as the edges of the iPSC colonies start to

curl up, the enzyme solution has to be aspirated and the cells washed. Extending the dispase treatment by even just 1–2 min can result in the colonies lifting of the dish surface and therefore in the loss of the cells.

5. We observed no difference in colony number or size between iPSCs obtained from scraping colonies with a 5 mL glass serological pipette or a cell scraper; we typically use cell scrapers because they are easier to use.
6. We observed that pipetting human iPSC colonies with glass serological pipettes (rather than plastic pipettes) leads to larger cell aggregates and better survival of the cells during passaging and the freeze/thawing procedure.
7. If at this stage a transfer of the human iPSCs onto Matrigel is required, the cells can be plated onto dishes precoated with Matrigel as described in Sect. 3.5.1. We observed that since the CTK treatment so efficiently eliminates the SNL feeder cells preadsorption of SNL cells on gelatin before the transfer of the human iPSCs to Matrigel is not necessary.

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