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A Protocol for Culture and Characterization of Human Induced Pluripotent Stem Cells after Induction

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Abstract

Human induced pluripotent stem cells (hiPSCs) are characterized by unlimited self-renewal and capability to differentiate into all three germ layers, that have potential to further differentiate into all types of cells and tissues. Human iPSCs retain all genetic information from their original donors and can be developed into disease models to study disease pathophysiology, identify disease phenotypes and biomarkers, and evaluate therapeutic efficacy and toxicity for drug development. Human iPSCs can also be used to develop cell therapies and regenerative medicine. In the last decade, the technologies for hiPSC generation and differentiation have advanced rapidly. Human iPSC culture and propagation are tedious and require careful handling. High quality hiPSCs are necessary for downstream applications. The methods, techniques, and skills for hiPSC maintenance and characterization are very different from those for immortalized cell lines. It will be a challenge for new laboratory staff, and sometimes even for experienced staff, to properly culture and maintain the high quality. Here, we describe a comprehensive protocol for hiPSC propagation in a chemically defined and feeder free culture condition. This step-by-step protocol describes all the reagents and experimental procedures in detail for culturing hiPSCs. This protocol also describes the experimental methods for hiPSC characterization including immunofluorescent staining and flow cytometry analysis with a panel of pluripotency markers, teratoma formation assay for validation of *in vivo* pluripotency, and Sendai virus detection to ensure elimination of the viral vectors. This protocol has been successfully used in our laboratory for hiPSC expansion and propagation, and is a useful reference guide for laboratory staff to work on hiPSC culture.

Basic Protocol 1: Propagation and cryopreservation of hiPSC cultures

Basic Protocol 2: Recovery of cryopreserved hiPSCs

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CONFLICT OF INTEREST STATEMENT:
Authors declare no conflict of interest.

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Basic Protocol 3: Examination of expression of pluripotency markers via immunocytochemical analysis

Alternate Protocol 1: Determination of expression of pluripotency markers via flow cytometry analysis

Basic Protocol 4: Pluripotency assessment via *in vivo* teratoma formation assay

Basic protocol 5: Examination of clearance of Sendai viral vector via reverse transcription PCR

Keywords

Human Induced pluripotent stem cells; hiPSC culture; hiPSC passaging; hiPSC characterization; hiPSC cryopreservation; teratoma

INTRODUCTION:

Induced pluripotent stem cells (iPSCs) are generated in vitro by the reprograming of somatic cells via the ectopic expression of a defined set of factors including octamer binding transcription factor 3/4 (Oct3/4), sex determining region Y—box 2 (Sox2), Krüppel-like factor 4 (Klf4), and cellular-Myelocytomatosis (c-Myc) (OSKM) (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). When properly maintained in cell culture, iPSCs have capabilities of unlimited passaging *in vitro* and differentiation into all cell types and tissues. Earlier methods for the generation of hiPSCs used retroviral vectors to transfect host cells with the transcription factors that integrated into the host genome. These reprogramming strategies have disadvantages such as low reprogramming efficiency and safety concerns for clinical applications. To ensure increased safety for clinical applications, nonintegrative systems have been developed to deliver the transcription factors via plasmids, proteins, self-replicating vectors, and RNAs. With these approaches, a variety of somatic cells have been successfully reprogrammed into hiPSCs with improved efficiency. The generation of hiPSCs from somatic cells has opened exciting opportunities not only for basic research, but also for therapeutics development. The reported applications of hiPSCs include *in vitro* disease modeling, drug screening, cell therapy, and regenerative medicine (Inoue et al., 2014; Shi et al., 2017). In particular, the generation of patient-specific hiPSCs that retain the patient's genetic information has offered the unique opportunity to study disease pathogenesis, identify disease phenotypes and biomarker sets, discover potential drug targets, and model diseases for drug development. Furthermore, the feasibility of differentiating hiPSCs into cells of all the three germ layers enables the development of tissues and organs for regenerative medicine. However, several important issues remain before hiPSCs can be widely utilized toward broad downstream applications. Some common issues include inappropriate cell expansion and maintenance of hiPSCs, resulting in poor quality hiPSCs with inefficient and inconsistent differentiation potential, and/or predisposition to mutations/abnormalities during long term cell culturing (Inoue et al., 2014; Shi et al., 2017). The ability to obtain and maintain high quality iPSCs is critical to ensure the successes of downstream applications. It is also important to periodically monitor and examine the quality and characteristics of hiPSCs maintained in laboratories for quality control purposes.

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Here, we present detailed, step-by-step protocols with specific reagent information for culturing and maintaining high quality hiPSCs. Basic Protocol 1 outlines procedures to maintain, expand, and cryopreserve hiPSCs. Basic Protocol 2 describes a method to initiate cultures of hiPSCs from frozen stocks. Basic Protocol 3 outlines steps to examine the expression of pluripotency markers using an immunofluorescence assay. Alternate Protocol 3 describes the examination of the pluripotency markers using flow cytometry. Basic Protocol 4 provides experimental steps for evaluating pluripotency via *in vivo* teratoma formation assay. For completeness, Basic Protocol 5 provides a method to detect Sendai viral vectors in hiPSCs using a reverse transcription PCR (RT-PCR) approach to validate the clearance of Sendai virus vectors used in the hiPSC induction.

It is worth noting that the reprogramming process and hiPSC maintenance may cause cell stress, resulting in genomic instability including unwanted genomic lesions and chromosomal abnormalities. A karyotyping examination should be routinely performed in the early passages (passages 7 to 10) and propagation (every 10~15 passages) to ensure genetic integrity of the hiPSCs (Figure 1). The hiPSC clones with an abnormal karyotype should be discarded. Meanwhile, as a rule of thumb for all cell cultures, cell line authentication testing (short tandem repeat (STR) profiling) and mycoplasma testing should be regularly conducted during long periods of hiPSC culturing (Figure 1).

STRATEGIC PLANNING

This protocol is mainly used for culturing hiPSCs for cell expansion, cryopreservation, and characterization after hiPSCs are generated or obtained. Culturing hiPSCs usually takes several months, which is time consuming and labor intensive. It is very important to start with high quality hiPSCs. If the original hiPSCs, generated or obtained, are of poor quality, the hiPSCs may not grow well in culture, which wastes time and resources. Before starting hiPSC culturing, the user should have good experience or training in regular cell line culture and be proficient with aseptic technique. Because no antibiotics are usually used in the hiPSC culture medium, the user's ability to maintain sterility in cell culture is a prerequisite for carrying out the following hiPSC culture protocol. It is important that the user has good cell/tissue culture experience because hiPSCs are more fragile than immortalized cell lines and need to be handled with special care and caution. Additionally, the user should read the following protocols and plan the experiments accordingly. All reagents, media and plasticwares for hiPSC culture should be purchased in advance. Equipment and hardware for the cell culture laboratory need to be well maintained and regularly certified. In additional to the following hiPSC culture protocol, any specific information for the given hiPSC line should be read and incorporated into the following protocol if needed. Because a daily medium change is usually required for most hiPSC lines, the user should plan their working schedule accordingly, and be sure to change the medium daily on weekdays and at least once over the weekend. We highly recommend using a non-enzymatic method to dissociate hiPSCs from the culture vessels because hiPSCs are fragile and prone to apoptosis. We use Versene solution (a specially formulated EDTA solution) for hiPSC dissociation, which gently dissociates cells from culture vessels without the use of enzymes. Due to the long cell culture process (1–3 months at least), hiPSCs may undergo changes that affect their abilities to self-renew or differentiate. Therefore, it is important to characterize each hiPSC

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line and to validate the unique features of hiPSCs during the hiPSC propagation. A set of experimental methods for hiPSC characterization and quality control are also included. For these hiPSC characterization experiments, the user should have knowledge and experience of the assays and purchase all the reagents in advance. If some experiments described below cannot be done in the user's laboratory, outsourcing of these experiments to collaborators' laboratories or commercial laboratories should be considered.

Basic Protocol 1: PROPAGATION AND CRYOPRESEVATION OF hiPSCs

Essential 8 (E8) Medium was originally developed by the laboratory of Dr. James Thomson (Chen G. et al 2011) based on the mTeSR1 medium, one of the first chemically defined medium for hiPSCs culturing under feeder-free condition. The medium contains eight essential components required to support hiPSC and hESC growth and expansion, providing a simpler medium for pluripotent stem cell propagation.

The propagation and culture of hiPSCs and hESCs with E8 Medium requires the use of Matrigel matrix to aid cell attachment and expansion. The Matrigel matrix is a mixture of extracellular matrix proteins derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, which contains laminin, collagen IV, heparin sulfate proteoglycans, entactin/nidogen, and several growth factors. Alternative coating matrices that have been reported to effectively support the culture of iPSCs include Geltrex, Vitronectin XF™, and CellAdhere™ Laminin-521.

Human iPSCs are more sensitive to enzymatic dissociation and prone to apoptosis and death, which often leads to poor survival rate during hiPSC propagation. The use of an EDTA-based enzyme-free dissociation method significantly improves cell survival and replating efficiency during hiPSC culture and propagation (Beers et al., 2012). With standard seeding density, the majority of hiPSC lines reached 70– 80% confluence within 4–5 days and can be continually passaged or frozen down (Cheng et al., 2019; Long et al., 2016). Any spontaneously differentiated cells observed under the microscope should be removed from the culture before passaging.

Cryopreservation is important to safeguard the valuable hiPSC lines. It is recommended to cryopreserve hiPSCs at earlier passages during cell propagation. A large batch of hiPSC frozen stock should be made and stored at −150°C (or liquid nitrogen) once the hiPSCs have been fully characterized. Cryopreserved hiPSCs with intermediate passage numbers are useful back-up resources for future expansion, in case of unexpected cell death or contamination issue; In addition, cryopreserved hiPSCs in frozen vials can also be immediately used in downstream applications such as the RNAseq analysis and mass spectrum based proteomic profiling.

This protocol describes the steps for routine culture and cryopreservation of hiPSCs which have been successfully used in our laboratory for over 100 hiPSC lines in the last 3 years. The hiPSCs were cultured and maintained in a feeder-free condition with chemically defined medium. The cells exhibit characteristics of high-quality hiPSCs based on growth rate,

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genetic stability, morphology, and phenotypes. They also maintain the ability to differentiate into three germ layer tissues *in vivo* (Chen et al., 2011).

Materials

Matrigel, hESC-Qualified Matrix, LDEV-free (Corning, cat. No. 354277), stored at −80°C

DMEM-F12 (Gibco, cat. No. 11320033)

HEPES (Gibco, cat. No. 11330-032)

Confluent hiPSCs (at 70 – 80% confluence). If iPSCs from stock vials are used (stored in the nitrogen storage container or −150°C deep freezer), go to Basic Protocol 2

Essential 8 Flex Medium kit (ThermoFisher Scientific, cat. No. A2858501)

Versene Solution (ThermoFisher Scientific, cat. No. 15040066), stored at 4°C

Y-27632 dihydrochloride (Tocris, cat. No. 1254) aliquot and store at −20°C

CryoStor CS10 (StemCell Technologies, cat. No. 7930), stored at 4°C

Chilled 1.5 ml Eppendorf tubes

50 ml conical tube (Falcon, cat. No. 352098)

Costar 6-well clear TC-treated multiple well plate (Corning, 3506)

Laminar flow hood

Phase-contrast microscope

Costar 2 mL aspirating pipettes (Corning, cat. No. 9186)

15-ml conical tubes

37°C, 5% CO_2 humidified incubator

Cryogenic tubes (Corning, cat. No. 430488)

Corning CoolCell Containers (cat. No. 432000)

Liquid nitrogen cell storage container or −150°C deep freezer

Protocol steps

Preparation of Matrigel coating

1. Matrigel arrives in 10ml bottles with varying concentrations across lots. Check the protein concentration of Matrigel on the label before working with it. Matrigel coating of 6-well plates for hiPSC culture uses 8.7 μg/cm^2 of the Matrigel (0.1mg/ml, 1 ml/well to coat 6-well plates). Aliquot the desired volume of Matrigel stock solution into Eppendorf tubes (e.g., 250 μl/vial) depending on

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the number of plates used in a typical experiment and store them at −20 °C. *Tips: the day before aliquoting the Matrigel stock, pre-chill all materials including Eppendorf tubes, racks, and pipette tips to 4 °C in a refrigerator. In addition, take out the Matrigel stock bottle from the freezer and submerge it immediately in ice, and let it thaw at 4 °C overnight. Aliquoting of Matrigel should always be performed on ice.*

2. On the day of coating, thaw an aliquot of Matrigel on ice. Add a small volume (300 to 500 μl) of ice-cold sterile DMEM-F12 medium to dilute the Matrigel aliquots. Since the Matrigel matrix will gel rapidly at 22 to 35 °C, *dilute Matrigel with a small amount of ice-cold sterile DMEM-F12 medium (0 to 4°C) first to avoid directly handling highly concentrated Matrigel.*

3. Transfer the diluted Matrigel aliquot to a pre-chilled 50 ml conical tube containing a volume of ice-cold DMEM-F12 to reach a final concentration of 0.1 mg/ml as the Matrigel coating solution. The dilution volume is calculated for each lot of Matrigel based on the protein concentration. *The detailed information is listed in the Certificate of Analysis for Matrigel on the Corning website.*

4. Pipette the Matrigel coating solution (0.1 mg/ml Matrigel in DMEM-F12 medium) into the wells of 6-well plates at 1ml/well. To coat 96-well plates, add 0.1 ml/well Matrigel coating solution.

5. Incubate the 6-well plates with Matrigel coating solution at room temperature (RT) for at least 1 h. After incubation, *Matrigel coated plates can be used immediately or stored at 4°C for up to one week prior to use. For storage, seal the plate (containing the Matrigel coating solution) with parafilm to prevent dehydration. The stored Matrigel coated plates should be placed in the tissue culture hood prior to use to pre-equilibrate to RT.*

Dissociation of hiPSCs for passaging

6. Once the hiPSCs reach 70 – 80% confluence, examine them under a tissue culture microscope to find any spontaneous differentiated cells (shown on Figure 2).

7. Remove differentiated cells by marking them under microscope and scratching them off by using a P20 pipette with pipette tips. This is especially crucial when there is an excessive number of differentiated cells.

8. Remove medium from the wells containing hiPSCs with an aspirating pipette and rinse the wells once quickly with Versene solution (1 ml/well in a 6-well plate). Versene solution is an EDTA solution that gently dissociates cells without the use of enzymes.

9. Add Versene solution to the wells (1 ml/ well in a 6-well plate). Gently swirl the plate to cover the entire well surface.

10. Incubate the 6-well plate at RT for 3–4 min. When cells start to separate and round up that form cell colonies and holes when observed under the microscope),

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the cells are ready to be removed from the wells. *This process can take longer than 5 min for certain cell lines or in larger culturing vessels.*

11. Carefully aspirate the Versene solution with an aspirating pipette.*Proceed to cryopreservation (Step 18b), or continue passaging the cells in 6 well plates as described below.*

12. Gently squirt 500 μl to 1 ml/well RT pre-warmed complete E8 Medium containing 10 μM Y-27632 hydrocholoride (final concentration) into the wells. To make complete E8 Medium, thaw 10 ml 50x supplement from the kit (stored in −80°C) in a 4°C refrigerator overnight, and then add it to 490 ml E8 Medium. This complete E8 Medium can be stored at 2–8°C for 2 weeks. Y-27632 dihydrochloride (Tocris, catalog number 1254) is an inhibitor of Rho-associated, coiled-coil containing protein kinase (ROCK). To prepare Y-27632 dihydrochloride stock solution, dissolve the powder in dH_2O to a 10 mM solution, then aliquot and store at −80°C (up to 1 year). Add 10 μl of 10 mM Y-27632 dihydrochloride to 10 ml complete E8 Medium. Collect cell aggregates from the 6-well plates in a 15-ml conical tube as follows.

   a. Cell aggregates should be appropriately sized for plating (10~30 cells per aggregate). *Little or no extra pipetting is required to break up cell clumps after Versene solution treatment.*

   b. Work with multiple wells at a time and work quickly to collect cells after adding E8 Medium to the well(s).

13. Aspirate the Matrigel coating solution from the Matrigel coated 6-well plates (if taking out from 4°C storage, the Matrigel coated plates need to be equilibrated to RT for 1 h) and add 1 ml RT warmed complete E8 Medium (with 10 μM Y-27632 dihydrochloride) into the 6-well plate immediately. (The Matrigel coated plates are from Step 5).

14. Determine the numbers of wells and the split ratio for seeding the cell aggregates (from Step-12) at the desired density into Matrigel coated wells containing complete E8 Medium with Y-27632. If the hiPSCs are younger than passage-10, the culture can be split every 4–5 days at a 1:2 to 1:4 ratio. If the hiPSCs are older than 10 passages, the colonies can be split at 1:6 to 1:10 every 4–5 days. The final volume of complete E8 Medium is 2 ml/well in 6-well plates.

15. Move the plate in several figure-eight motions (slow and gentle movements) to evenly distribute cells across the surface of the wells.

16. Gently place the culture plate into a 37°C, 5% CO_2 incubator and incubate the cells overnight.

17. On the next day, replace the old medium with 2 ml/well fresh complete E8 Medium without Y-27632, followed by medium change daily with 2 ml/well fresh complete E8 Medium without Y-27632 (usually for 4 to 5 days). *It is normal to see cell debris and small colonies under the microscope the day after passaging. It is important to carefully monitor cell morphology using a*

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microscope every day during medium changes and to remove any differentiated cells.

18. Keep passaging the hiPSCs until they reach 70–80 % confluence. The hiPSC colonies are generally ready to passage after 4 to 5 days in culture. If there is a need for the cryopreservation of the hiPSCs, the hiPSCs should be denser than 80% confluent for this purpose.

    a. For hiPSC passaging (when the cells reach 70–80% confluence), use the above-described steps (steps 6–17).

    b. For hiPSC cryopreservation, dissociate the cells with Versene solution when the cells reach ~80 % confluence as I step 6–11. Aspirate the spent medium and then gently add CryoStor™ CS10 cell cryopreservation medium into the wells (700 μl/well in a 6-well plate). Dislodge cells by gently pipetting the medium up and down. Aliquot 350 μl/vial into separate cryogenic vials (hiPSCs from one well of a 6-well plate are stored into 2 vials). Place the vials in a Corning CoolCell freezing container and store at −80°C for at least overnight (or 1–3 days). Then, transfer the vials from the Corning CoolCell freezing container into a liquid nitrogen tank or −150°C deep freezer for long-term storage.

    c. Human iPSCs at 70–80% confluence can also be used for experiments including hiPSC characterization (Basic Protocols 3, 4, and 5), and other downstream hiPSC applications (e.g., differentiation to neurons, hepatocytes, and cardiomyocytes, and various organoids).

Basic Protocol 2: RECOVERY OF CRYPRESERVED hiPSCs

This protocol describes a centrifugation-free method to recover cryopreserved hiPSCs, which highly improves hiPSC survival and recovery rate upon thawing. After thawing a vial of frozen hiPSCs (from nitrogen tank or −150°C deep freezer) in a 37°C water bath, all the contents in the vial are transferred into one well of a pre-coated 6-well plate and incubated for 60 min in the presence of 10 μM Y-27632 dihydrochloride. After confirming attachment of hiPSC colonies to the plate, the medium is removed and replaced with a fresh complete E8 Medium containing 10 μM Y-27632 dihydrochloride.

Materials

Frozen stock of hiPSCs

70% ethanol

Dry ice

Matrigel-coated, pre-equilibrated, 6-well plate (prepared as described in Basic Protocol 1, Step 1 to 5)

Water bath

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DMEM-F12 (Gibco, cat. No. 11320033)

HEPES (Gibco, cat. No. 11330-032)

Essential 8 Flex Medium kit (ThermoFisher Scientific, cat. No. A2858501)

Versene Solution (ThermoFisher Scientific, cat. No. 15040066), stored at 4°C

Y-27632 dihydrochloride (Tocris, cat. No. 1254) aliquot and store at −20°C

CryoStor CS10 (StemCell Technologies, cat. No. 7930), stored at 4°C

Chilled 1.5 ml Eppendorf tubes

50 ml conical tube (Falcon, cat. No. 352098)

Costar 6-well clear TC-treated multiple well plate (Corning, 3506)

Laminar flow hood

Phase-contrast microscope

Costar 2 mL aspirating pipettes (Corning, cat. No. 9186)

15-ml conical tubes

37°C, 5% CO2 humidified incubator

Cryogenic tubes (Corning, cat. No. 430488)

Corning CoolCell Containers (cat. No. 432000)

Liquid nitrogen cell storage container or −150°C deep freezer

Protocol steps:

1. Remove a cryopreserved vial of hiPSCs (prepared in Basic Protocol 1, step 18 or purchased) from liquid nitrogen storage or −150°C freezer and immediately place it into a 37°C water bath (using dry ice to transfer from the freezer to the 37°C water bath).
2. Thaw the cells by gently swirling the vial in the 37°C water bath until only a small piece of ice remains in the vial (just before it thaws completely).
3. Transfer the vial to a laminar flow hood and wipe its surface with 70% (vol/vol) ethanol before opening. *If the vial is labeled with a marker, record the information listed on the label before wiping with 70% ethanol.*
4. Aspirate the Matrigel containing solution from the Matrigel coated 6-well plate (pre-equilibrated to RT for 1 h in the hood), and immediately add 5 ml/well of the RT pre-warmed complete E8 Medium with 10 μM Y-27632 dihydrochloride. (Matrigel-coated plate from basic protocol, Step 5).

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5. Transfer the thawed hiPSCs from the vial to the prepared 6-well plate dropwise, evenly distributing them into the complete E8 Medium in the well.

6. Move the plate in several figure-eight motions (slow and gentle movements) to distribute cells evenly across the surface of the bottom of 6-well plates.

7. Gently place the 6-well plate into a 37°C, 5% CO_2 incubator and incubate the cells for 1 h.

8. Examine cell attachment under a microscope to make sure most cells have attached to the well. If not, extend the incubation time for an additional 30 min (hiPSCs may have variations in attachment rates).

9. Carefully remove the supernatant (containing freezing medium) from the plate and add 5 ml/well of RT pre-warmed fresh complete E8 Medium with 10 μM Y-27632 dihydrochloride.

10. Place the plate into the 37°C, 5% CO_2 incubator and incubate the cells overnight.

11. On the next day, remove the medium (containing Y-27632 dihydrochloride) and replace with 2 ml/well complete E8 Medium without Y-27632 dihydrochloride. Return the plate to the 37°C, 5% CO2 incubator.

12. After the second day post-thawing, remove the old medium and feed the hiPSCs with 2 ml/well fresh complete E8 Medium daily (medium change daily). Monitor and assess cells growth until they reach 70 – 80% confluence for splitting and passaging (approximately 2 – 5 days after thawing). *It is normal to see cell debris and small colonies after thawing. The cells are now ready to use for characterization (Basic Protocols 3, 4, and 5), cryopreservation, or other experiments of interest.*

13. Continue hiPSC culture and passaging as described in Basic Protocol 1, Step 6 to 18.

Basic Protocol 3: Validation of pluripotency of hiPSCs with IMMUNOCYTOCHEMICAL ANALYSIS

Human iPSCs possess unique properties of self-renewal and differentiation into all of the cell types in our body. However, spontaneous differentiation occurs frequently in hiPSC propagation, which if left unchecked, can reduce hiPSC self-renewal, and/or differentiation into the intended cell types. Phenotypical characterization of hiPSCs by using a panel of pluripotency markers is a standard method to efficiently qualify hiPSCs. This protocol describes how to evaluate the expression of pluripotency markers in hiPSCs using immunocytochemistry. The hiPSCs are plated, stained, and analyzed using a microscope in 96-well plate format. Immunocytochemistry provides visualization of pluripotency markers in individual cells within a colony, allowing assessments of subcellular localization of each marker. This analysis should be performed after the removal of transgenes (usually after 10 passages) and before downstream applications such as differentiation into neurons or liver organoids.

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Materials:

Confluent hiPSCs in 6-well plates (from Basic Protocol 1, step 18c)

Matrigel-coated, pre-equilibrated, 96-well plate (prepared as described in Basic protocol 1, Step 1 to 5)

DPBS (with calcium and magnesium, ThermoFisher Scientific, 14040141)

32% (w/v) Paraformaldehyde (Electron Microscopy Sciences, 15714-S)

Triton X-100 (Sigma-Aldrich, T8787)

Blocking solution (Cell Staining Buffer, BioLegend, 420201)

Hoechst 33342 (Invitrogen, cat. No. H1399)

DPBS (without calcium and magnesium, ThermoFisher Scientific, cat. No. 14190144)

96-well plate (Greiner Bio-one, 655090)

Protocol Steps

Day 1

1. Prepare a Matrigel coated 96-well plate by adapting Basic Protocol 1 steps 1–5 (Adjust volumes by adding 100 μl/well diluted Matrigel coating solution (0.1 mg/ml Matrigel in DMEM-F12 medium)) and incubate at RT for 1 h in the hood.

2. After dissociating hiPSC colonies using Versene solution (following Basic Protocol 1 steps 6–12), take the cells from one well in a 6-well plate that are added to 5.5 ml of complete E8 Medium supplemented with 10 μM Y-27632 dihydrochloride to make up 6 ml of the dissociated hiPSCs.

5. Seed 100 μl/well of the diluted hiPSC suspension into the Matrigel-coated 96-well plate after removing the Matrigel coating solution from the 96-well plate. The number of wells for hiPSC seeding is dependent on the experiment needs.

6. Examine under the microscope to confirm the hiPSC seeding and incubate at 37°C, 5% CO2 incubator overnight.

Day 2

7. Change the medium by removing old medium and add 100 μl/well of RT prewarmed complete E8 Medium without Y27632.

Day 3

8. Check the cells under the microscope to ensure that they are 30–50% confluent. If that’s the case, remove medium and fix the cells by adding 100 μl/well of 4% paraformaldehyde diluted in DPBS (with calcium and magnesium). Incubate the 96-well plate for 15 min at RT.

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9. After hiPSC fixation, wash the cells three times with 100 µl/well of DPBS (with calcium and magnesium), each for 5 min leave the DPBS in wells for 5 min.
10. Permeabilize cells by adding 100 µl/well of 0.5% triton X-100 in DPBS (with calcium and magnesium). Incubate for 15 min at RT.
11. Aspirate the solutions and wash the cells three times with 100 µl/well of DPBS (with calcium and magnesium) each for 5 min (leave the DPBS in wells for 5 min).
12. Aspirate the solution, add 100 µl/well of Blocking solution, and incubate the plate for 1 h at RT.
13. Aspirate the blocking solution and add a single primary antibody at 100 µl/well (with the appropriate dilution in Blocking solution for the antibody listed in Table 1). Use 100 µl/well of blocking solution without antibody as negative control wells. Usually, 3 wells are needed for each antibody dilution and 3 wells for the negative control in each experiment.
14. Incubate the plate overnight at 4°C.

Day 4

15. Aspirate the solution and wash the cells three times with 100 µl/well of DPBS (with calcium and magnesium, each for 5 min (leave the DPBS in wells for 5 min).
16. Aspirate the wash buffer, add secondary antibody diluted in blocking solution at the appropriate concentration (see Table 1), and incubate at RT in the dark for 1 h.
17. Aspirate the solution and wash the cells three times with 100 µl/well of DPBS (with calcium and magnesium), each for 5 min (leave the DPBS in wells for 5 min).
18. Add 100 µl/well of 1 µg/ml Hoechst 33342 in DPBS (with calcium and magnesium) and incubate for 15 min at RT in the dark.
19. Aspirate the solution and wash the cells three times with 100 µl/well of DPBS (with calcium, magnesium), each for 5 min (leave the DPBS in wells for 5 min).
20. Add 100 µl of DPBS (with calcium and magnesium) to each well.
21. Image cells with the appropriate filters (10 X objective, 6 images per well).

Samples of images are shown in Figure 3a.

Alternate Protocol 1: Examining the expression of pluripotency markers via flow cytometry analysis

Flow cytometry is an alternative way to quantitatively examine the expression of pluripotency markers in hiPSCs. This method can quantitate pluripotency marker expression in cell populations, and flow cytometry analysis provides more quantitative data analysis

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compared to the immunocytochemistry method, whereas the immunocytochemistry analysis using fluorescence microscopy provides qualitative information such as subcellular localizations of the markers. The following protocol outlines the steps and reagents for flow cytometry analysis. Cells in single cell suspension is required for flow cytometry, thus, an enzymatic dissociation method is used.

Materials:

Confluent hiPSCs (70 ~ 80% confluency) in 6-well plates (from Basic Protocl-1, step 18c)

DPBS, calcium, magnesium (ThermoFisher Scientific, 14040141)

32% (w/v) Paraformaldehyde (Electron Microscopy Sciences, 15714-S)

Blocking solution (Cell Staining Buffer, BioLegend, 420201)

TrypLE Express Enzyme (ThermoFisher Scientific, 12605010)

Fetal bovine serum (FBS) (vendors may vary depending on availability, e.g., HyClone Characterized FBS, US Origin, SH30071.03IH30-45)

Tween-20 (Sigma-Aldrich, P9416)

DPBS, no calcium, no magnesium (ThermoFisher Scientific, cat. No. 14190144)

Flow cytometry (Agilent, NovoCyte Penteon)

Protocol Steps:

1. Dissociate hiPSCs by using 1 ml/well of TrypLE Express enzyme solution with the standard cell dissociation method (using the hiPSCs with 70–80 % confluence cultured in 6-well plates described in the Basic protocol 1).
2. Collect the cells from one well into a 1.5 ml Eppendorf tube, centrifuge (200 x g, 5 min) to pellet the cells, and wash once with 1 ml of DPBS (without calcium and magnesium).
3. Aspirate supernatant from the 1.5 ml tube.
4. Fix the cells: Add 0.5 ml/tube of 4% paraformaldehyde diluted in DPBS (without calcium and magnesium) to resuspend the cell pellet, and incubate for 10 min at RT.
5. Wash the cells twice with 1 ml/tube of DPBS (without calcium and magnesium), by centrifuging at 200 x g to pellet cells.
6. Resuspend cells with 0.5 ml/tube of permeabilization buffer and incubate for 10 min at RT.
7. Aspirate 0.5 ml permeabilization buffer from the 1.5 ml tube after centrifugation (200 g)

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8. Add 0.5 ml/tube of the desired fluorophore-conjugated antibody and use a nonspecific rabbit or mouse IgG as a negative control (see Table 2). (Typically, use only one antibody per tube)

8. Incubate the cells for 1 h at 4°C on a shaker (rotating at 20 RPM). Wrap the vial with aluminum foil to avoid light exposure.

9. Wash the cells twice with 1 ml DPBS (without calcium and magnesium), by centrifuging at 200 x g to pellet cells.

10. Analyze stained cells using a flow cytometer with the appropriate wavelengths based on the antibody labeling. Collect at least 10,000 events from the cell gate.

The representative data are included in Figure 3b.

Basic Protocol 4: PLURIPOTENCY ASSESSMENT VIA IN VIVO TERATOMA FORMATION

By definition, pluripotency refers to a cell's ability to differentiate into all cell types of the body. Cell morphology, growth property, karyotype, and epigenetic markers may provide some clues on whether the cells are likely pluripotent, but they are indirect and unreliable. Random differentiation through embryoid body formation or directed differentiation into specific lineages can in theory address this question, but such in vitro culture methods are prone to false conclusion due to artificial effects caused by inconsistent reagents or poor technical skills. Conversely, injecting hiPSCs into immunocompromised mice to test their ability to form teratomas is a simple and reliable method to find out whether a particular hiPSC line is capable of differentiating into a variety of tissues, including cell types that originate from all three germ layers (ectoderm, mesoderm, and endoderm) during normal embryonic development.

In addition to confirming whether a particular cell line is truly pluripotent in a qualitative manner, teratoma formation assay can also provide semi-quantitative insights on a cell line's differentiation propensity. Not all hiPSC lines have equal differentiation tendency towards all three germ layers, as the original source of the hiPSCs and reprogramming method can significantly influence differentiation propensity. An abundance of tissues from one particular germ layer formed in teratomas may hint at whether the hiPSC line is a suitable choice for in vitro differentiation into the intended cell type for further studies or disease modeling.

Since teratoma assays involve the use of laboratory animals, these experiments need to be approved by the Institutional Animal Care and Use Committee (IAUCU).

Materials:

hiPSCs (~80% confluence) in 6-well plates (see Strategic Planning)

Essential 8 medium supplemented with 25 mM HEPES (pH 7.4).

Matrigel, hESC-Qualified Matrix, LDEV-free (Corning, cat. No. 354277), stored at −80°C

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Two 8- to 26-week old NSG mice (JAX Stock NO. 005557), both male and female mice can be used.

Cell counter (Nexcelom Auto T4)

Centrifuge (Beckman Allegra X-14R)

Isoflurane vaporizer (SurgiVet Classic T3TM Vaporizer)

Isoflurane liquid (Baxter Forane #10019-360-40)

Tuberculin Syringe (1ml, BD REF-309659)

Hypodermic needle (25G X 5/8", BD REF-305122)

Scissors (Roboz RS-6808)

Forceps (Roboz RS-5159)

10% Neutral Buffered Formalin (BDH #BDH0502-4LP)

1.5 ml Eppendorf tubes

50 ml conical tubes

Ice

Protocol steps:

1. Harvest 70–80% confluency hiPSCs from one 10-cm culture dish or all six wells of a 6-well plate using Versene, as described in Basic Protocol 1, Step 6–12.

2. Pellet the cells at RT for 5 min at 300 x g. Aspirate the supernatant and resuspend the pelleted cells in 500 μl of complete E8 Medium supplemented with 25 mM HEPES (pH 7.4).

3. Count cells and transfer 8×10^6 cells into a sterile microcentrifuge tube. Adjust the total volume to 400 μl by adding additional E8 Medium with 25 mM HEPES (pH 7.4). Cool the cell suspension on ice.

4. Thaw an aliquot of Matrigel on ice. Mix 200 μl of Matrigel with the cold cell suspension so that the final Matrigel concentration is 33% (v/v). Store the tube on ice.

5. Turn on the gas tank regulator to supply pure oxygen to the isoflurane vaporizer. Set the isoflurane concentration to 4–5%.

   It is important to minimize leakage of isoflurane into the procedure room during the entire process. An approved gas scavenge system should be used to collect used isoflurane vapor, and the entire anesthetic station should be certified annually.

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6. Place two immunocompromised NSG mice into the induction chamber of the vaporizer.

7. Attach a 25G needle to a 1-ml sterile syringe and draw the entire 600 μl of cell suspension from Step 4 into the syringe.

8. Take one anesthetized mouse out of the isoflurane chamber. Lay the mouse on its back and place its muzzle inside the nose-cone of the isoflurane vaporizer.

9. Quickly inject 0.1 ml of cells subcutaneously into the groin area of one of its hind legs and then inject 0.1 ml of cells into the groin area of the other hind leg. Place the injected mouse back into its cage.

10. Take the second mouse out of the chamber and repeat the injection procedures described in Step 8–9 above.

It is important to inject both mice quickly to avoid warming of the cell suspension inside the syringe, which may lead to gelling of the Matrigel.

11. Turn off the isoflurane vaporizer and oxygen tank gas regulator.

12. Mark the cage card with cell line information and injection date. Injected mice should wake up in a few minutes. Return the cage to the animal room.

13. Check the injected mice on the next day and then on a weekly basis. When the tumors reach ~1 cm in diameter, monitor the mice every other day to make sure the tumors do not exceed the size limit (usually 2 cm in diameter) specified by the Animal Care and Use Committee and the specific animal facility.

The rate of tumor growth varies widely depending on the cell lines, but for most hiPSC lines, teratomas are ready to be harvested 6–12 weeks post injection.

14. When one or both tumors nearly reach the size limit, euthanize the mouse by CO_2 asphyxiation followed by cervical dislocation.

Protocols for euthanizing mice can be found in many books and protocols, such as the laboratory manual published by the Cold Spring Harbor Lab Press: Manipulating the Mouse Embryos, the 4th Edition, 2014, edited by R. Behringer, M. Gertsenstein, K.V. Nagy, and A. Nagy (ISBN 978-1-936113-00-2).

15. Lay the euthanized mouse on its back and spray 70% ethanol on the ventral side to wet the fur. Use a pair of sharp scissors to make a large cut (~2 cm long) in the skin of the abdomen area. Use gloved fingers to grasp each side of the cut and tear the skin apart to expose the teratomas, which should sit between the skin and muscle body wall (Fig 4).

16. Use a pair of iris forceps and a pair of scissors to carefully remove the teratomas and fix them in 10% Neutral Buffered Formalin in 50mL conical tubes.

17. Embed fixed teratomas in paraffin and section them to make 5 um-thick slides and then stain with H&E (hematoxyin and eosin) following standard procedures, *or* send them to a contract histology lab to perform these procedures.

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18. Examine the stained slides under a light microscope. Alternatively, the entire slide can be scanned using a digital slide scanner, such as the NanoZoomer scanner made by Hamamatsu, Inc. The goal is to look for tissues derived from various germ layers.
19. Truly pluripotent hiPSCs should give rise to tissues derived from all three germ layers. As shown in Fig 5, the most used tissue for representing ectoderm is the neural epithelium, the most reliable and obvious tissue for mesoderm is cartilage or bone, and the most typical endodermal tissue is the gut, such as intestine. Most properly derived hiPSC lines can give rise to all three germ layer tissues, but their differentiation propensities are often biased towards one or two germ layers, resulting in large differences in tissue abundancies for the three germ layers. This may be caused by epigenetic memories retained from the donor cell type used for hiPSC reprogramming, partial differentiation caused by improper culturing conditions, or mishandling prior or during the hiPSC injection process. When a particular cell line fails to yield tissues from all three germ layers, it is recommended to repeat the teratoma assay with a different hiPSC clone from the same reprograming event. If no other clones are available, subcloning the original hiPSCs may be able to identify good subclones by eliminating partially differentiated cells or cells that have accumulated deleterious mutations (such as abnormal karyotypes) during the culture process.

Basic protocol 5: EXAMINATION OF SENDAI VIRIAL VECTORS

The reprogramming of somatic cells via the Sendai virus delivery system has become a common approach to efficiently generate hiPSCs. The advantages of using Sendai virus reprogramming system include safety for humans as no human diseases are linked to this virus, broad cellular tropism as it binds to ubiquitous sialic acid as the cellular receptor to enter human cells, and a non-integrating viral replication mechanism that does not alter the host genome. Sendai virus mediated hiPSC induction has been shown to maintain genomic integrity with minimal level of genetic aberration. However, it still takes a few months for transgenes to be cleared from the reprogrammed cells. Validation of the clearance of reprogramming materials from hiPSCs is an important quality control for establishing hiPSC lines. Reverse transcriptase polymerase chain reactions (RT-PCR) with primers binding to the reprogramming factors is considered as one the best choices for detecting the transgenes. In this protocol, an RT-PCR analysis is used to confirm that no residual transgenes of viral origin remain in the hiPSCs when the non-integrative Sendai virus delivery system was used to generate hiPSCs. The original human somatic cells that are freshly transduced with the Sendai virus system are used as a positive control for this experiment. It is important to use hiPSCs that have cleared genetic materials from iPSC induction for other downstream applications such as differentiation into neurons or organoids.

Materials:

Confluent hiPSCs in 6-well plates (from Basic Protocl-1, step 18c)

Somatic cells (e.g., Fibroblasts)

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Complete somatic cell culture medium (e.g., DMEM, 10% FBS for fibroblasts)

TrypLE Express Enzyme (ThermoFisher Scientific, 12605010)

CytoTune™-iPS 2.0 Sendai Reprogramming Kit (ThermoFisher Scientific, A16517)

Qiagen RNeasy Plus Mini Kits (Qiagen, 74034) or other RNA isolation protocols

SuperScript™ III First- Strand Synthesis SuperMix (ThermoFisher Scientific, 18080400) or other reverse transcription protocols

Platinum II Hot-Start PCR Master Mix (ThermoFisher Scientific, 14000012) or other PCR protocols

DPBS, without calcium and magnesium (ThermoFisher Scientific, cat. No. 14190144)

PCR thermocycler

PCR primers (Table 3).

E-Gel Agarose Gels with SYBR Safe DNA Gel Stain, 1% (ThermoFisher Scientific, A45202)

E-Gel 1 Kb Plus DNA Ladder (ThermoFisher Scientific, 10488090)

E-Gel Sample Loading Buffer, 1X (ThermoFisher Scientific, 10482055)

Invitrogen E-Gel iBase and E-Gel Safe Imager (Fisher Scientific, 10001123)

G: Box Chemi-XX6 gel doc system (Syngene)

NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific)

Protocol steps:

Preparation of positive control samples

Day −2

1. Seed human fibroblasts into a well of a 6-well plate so that the cells reach 30–60% confluence on the day of experiment (Day 0).

Day 0

2. Change culture medium and add CytoTune™-iPS 2.0 Sendai reprogramming vectors at the appropriate MOI following the manufacturer’s instructions.

3. Incubate the cells overnight in a 37°C incubator with 5% CO_2.

Day 1

4. Aspirate medium containing Sendai reprogramming vectors in the 6-well plate. Add 1 ml fresh complete fibroblast medium and return to the incubator.

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5. Change medium every other day (1 ml/well).

Day 4

6. Dissociate cells using TrypLE (standard cell dissociation method) and collect the final 1 ml/well cells into a 1.5 Eppendorf tube.

7. Pellet the cells via centrifugation (200 x g) for 5 min. Remove the supernatant and wash the cell pellet once with 1 ml of DPBS (without calcium and magnesium).

8. Pellet cells again via centrifugation (200 x g) for 5 min. Remove the supernatant. The cell pellet can be used immediately for the experiment (continue to Step 12 below), or store at −80 °C for later use.

RT-PCR for reprogramming vectors

9. Collect hiPSCs from four confluent wells of a 6-well plate (70–80% confluence, following the Basic Protocol 1) into a 15ml conical tube by enzymatic dissociation method using 1 ml/well of TrypLE Express solution. Pellet cells via centrifugation (200 x g) for 5 min.

10. Resuspend the cell pellet with 1 ml DPBS (without calcium and magnesium). Transfer the cell suspension into a 1.5 ml Eppendorf tube. Pellet cells via centrifugation (200g) for 5 minutes. Wash the cell pellet once with 0.5 ml DPBS (without calcium and magnesium).

11. Pellet cells via centrifugation (200 x g) for 5 min and remove the supernatant. The cell pallet can be used immediately for the experiment as the following step, or be stored at −80°C for later use.

12. Extract total RNAs from the hiPSC sample (from Basic Protocol 1, Step 10) and Sendai virus transfected fibroblast pellet from Step 10 using Qiagen RNeasy Plus Mini Kit by following the manufacturer's instructions. Quantitate the RNA concentrations with a Nanodrop 2000 spectrophotometer, which provides ratio of 260/280 nm and RNA concentration (μg/ml) detected in samples. The typical ratio of 260/280 nm is 1.8–2.2 and RNA yield is in a range of 2.4–4.0 μg/ml.

13. Use 0.5 μg of total RNA to synthesize cDNA in a 20-μl reaction using the cDNA reverse transcription kit, SuperScript™ III First- Strand Synthesis SuperMix, following the manufacturer's instructions.

14. Add 1 μl of the cDNA synthesized above to 20 μl/tube of Platinum II Hot-Start PCR Master Mix for PCR. Prepare 5 reactions for each test sample, which includes one reaction of internal control (GAPDH) and four reactions using the primers listed in Table 3.

15. Run PCR with the following conditions: 2 min at 94 °C, followed by 30 cycles of 14 s 94 °C → 15 s at 60 °C → 15 s at 68 °C on a Mastercycler pro S (Eppendorf) Thermal Cycler instrument.

16. Take 10 μl of each PCR product and mix with 10 μl of 2 x loading buffer. Load each sample to one lane on a 1% agarose gel.

17. Load 25 μl of 1 Kb DNA ladder to a lane in each 1% E-Gel Agarose gel.

18. Run DNA gel with E-Gel 0.8–2% Program, and then stain with the SYBR Safe DNA Gel Stain to visualize the PCR products. Analyze PCR products using an imager (e.g., G: Box Chemi-XX6 gel doc system by Syngene). A typical gel picture is shown in Figure 3c.

COMMENTARY:

Background Information:

Pluripotent cells have the capability to differentiate to all somatic lineages in the human body. It has been found that the pluripotent cells exist transiently in the inner cell mass (ICM) of the blastocyte. The collection of these cells at this period and the generation of embryonic stem-cell (ESC) lines has successfully maintained pluripotency of these cells in culture. The ESCs that are propagated in a defined cell culture environment show a transcriptional profile and epigenetic states resembling those of pluripotent epiblast cells (Hanna et al., 2010b; Nichols and Smith, 2012; Weinberger et al., 2016). An additional source of pluripotent cells is the direct reprograming of somatic cells into pluripotency *in vitro* via the ectopic expression of defined transcription factors, generating induced pluripotent stem cells (iPSCs) (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). Advancements in hiPSC-related technologies have offered great opportunities for stem-cell-based therapies, tissue/organ regeneration/replacement, and disease modeling. Patient derived hiPSCs preserve the genetic background and disease-related gene mutations from the donors and have been successfully used as *in vitro* disease models for studies of disease pathophysiology and evaluation of drug efficacy for drug development. Modeling diseases using human cells (e.g., neurons, hepatocytes, and cardiomyocytes) and organoids (e.g., brain organoids, liver organoids etc.) derived from hiPSCs have advantages over animal models as many disease phonotypes are not recapitulated by animal models due to species differences (Farkhondeh et al., 2019). Patient derived hiPSCs could be especially useful for modeling genetic diseases, as there are currently over 7000 genetic diseases, many of which are rare diseases that lack proper disease models. The original process of hiPSC generation and expansion requires the use of mouse or human fibroblasts as a feeder layer to support hiPSC growth. The preparation of feeder cells is labor-intensive and contributes to significant batch variations, resulting in inconsistent hiPSC expansion. More recently, feeder free culture systems have been developed for hiPSC generation and expansion, consisting of Matrigel, or other extracellular matrix (ECM) proteins and completely defined stem cell culture medium. The system has been widely applied to maintain and culture hiPSCs and hESCs. Thus, it is important to update hiPSC culture protocols periodically to ensure a reliable and consistent process to culture and maintain hiPSCs in laboratories for downstream applications such as cell therapy development and disease modeling. In order to find the optimal culture condition for hiPSCs, we have evaluated and optimized parameters including matrix, cell culture medium, cell seeding density, dissociation reagent, and other related reagents shown in each section of this protocol.

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Culturing hESCs and hiPSCs is very challenging work that requires experienced staff with excellent cell culture skill and gentle hands. Poor post-thaw recovery rate, spontaneous differentiation, contamination with bacterial/fungi/mycoplasma, and lower reproducibility are some common issues encountered with hiPSC cultures. In addition to proper staff training for hiPSC culture work, many details and tips included in this protocol have been described to overcome these obstacles in hiPSC culture process. The low cryopreservation efficiency of hiPSCs has been improved recently by supplementing ROCK inhibitor in the cryopreservation medium and revival medium, although extensive technical improvements and skills are also required to achieve high recovery rate from cryopreserved stock. Eliminating centrifugation of hiPSCs after thawing increase hiPSC recovery rate as it reduces cell stress. The critical standards for high-quality hiPSCs are still in development by researchers in the field. The quality of hiPSCs can be influenced by many factors including culture materials, culture conditions, culture manipulation techniques, and operator's handling skills. We have included the parameters of high-quality hiPSCs in our protocol that are measured by colony morphology observed by microscopy, expression of pluripotency markers determined by immunofluorescence assay and flow cytometry analysis, transgene/vector free condition assayed by RT-PCR, and cell identity by STR (to ensure that the identity of the hiPSCs is the same as that of the original somatic cells). The combination of these parameters, cytogenetic analysis, and potential of three germ layer differentiation *in vivo* or *in vitro* should provide valuable information to examine the quality and integrity of hiPSCs in long term culture, that could easily last weeks to months.

In addition to confirming whether a particular cell line is truly pluripotent for differentiation to three layers in a more qualitative manner, the teratoma formation assay can provide semi-quantitative insights into a cell line's differentiation propensity. Not all hiPSC lines have an equal differentiation tendency towards all three germ layers, because the original source of the hiPSCs and reprogramming method can significantly influence iPSC differentiation propensity. Therefore, the abundance of tissues from one particular germ layer in teratomas may hint at whether the hiPSC line is a suitable choice for *in vitro* differentiation into a particular type of cells, tissues, or organs.

Critical Parameters:

Experiences in cell culture, sterile technique, and gentle handling are needed for staff who work on hiPSC culture. Careful monitoring of cell morphology is an important task during the hiPSC culturing process. Pluripotency is an important property of hiPSCs that allows hiPSCs to maintain unlimited self-renew and to differentiate into all the cell types of interest for various downstream applications. However, hiPSCs can spontaneously differentiate into other types of cells during culture and passaging. Careful monitoring of cell morphology and removal of differentiated cells are critical for maintenance of pluripotency for downstream applications after long-term culture.

Troubleshooting:

The following Table 4 lists common problems, their causes, and potential solutions for above-described sections of this protocol.

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Understanding Results:

The images of hiPSCs and results of hiPSC characterization are shown in Figures 2 and 3. More results of hiPSC culture, passaging, and characterization were also described in detail in our previous papers (Li et al., 2020; Xu et al., 2020). The following special points are useful for understanding the results and actions that may be taken.

Proper maintenance of hiPSCs in culture is critical for sustaining their ability of self-renewal and efficient differentiation into three germ layers. A critical step during hiPSC culture and propagation is to passage hiPSC cells at an appropriate cell density (confluency) and time. Daily evaluation of the hiPSC culture via microscopy is required to monitor the morphology and confluence of hiPSCs. Because each hiPSC line may exhibit a different growth rate, the split ratio of a given hiPSC line needs to be adjusted at each passage based on the appearance of the hiPSC colonies (confluency and days needed to reach 70–80% confluence). Usually, the split ratio of hiPSCs during passaging is between 1:6 and 1:10. When the hiPSC colonies are becoming too dense or too large, the higher split ratio (1:10 or higher) is needed. The cells will be passaged at every 4–5 days according to the appearance of colony’s size and density. Importantly, any spontaneously differentiated cells should be removed first before passaging.

Variations between cell lines are expected in hiPSC culture. Standardized quality control measures for hiPSC culture can minimize variability and increase reproducibility. The morphology of hiPSCs in culture is the most easily monitored indicator of cell quality, as it can be observed under a simple microscope. Typically, hiPSCs appear as relatively round colonies with defined border and tightly packed cells that have a high ratio of nucleus to cytoplasm (larger nuclear area than cytoplasm in a cell). The hiPSCs are characterized by specific pluripotency cell surface markers (SSEA-4 and TRA-1-60) and key transcription factors regulating the pluripotency network (SOX2, OCT4, and Nanog). Those cell surface markers and transcription factors should be homogeneously expressed in nearly all cells in the hiPSC colonies. In addition, high-quality hiPSCs exhibit no transgene (absence of the genes and gene delivery vectors used in generation of hiPSCs from somatic cells).

The teratoma assay is commonly used to evaluate the differentiation potential of hiPSCs *in vivo,* and tests whether hiPSCs can differentiate to all three germ layers. The hiPSC derived teratoma formed in an immunodeficient mouse is usually a heterogeneous tumor composed of terminally differentiated cells representative of all three germ layers. Teratomas are validated by H&E staining, and by the specific markers for endoderm, mesoderm, and ectoderm germ layers (Figure 5).

In addition, a few specific notes for the results are listed as follows.

1. Unexpected differentiation of hiPSCs observed during the culture and passaging. This may be due to an unhealthy hiPSC colony or disease (gene mutation) related mutations (if the hiPSCs were generated from patient cells). Reselecting a healthy hiPSC colony from the pool of hiPSCs generated or re-generation of hiPSCs from patient samples may resolve the unexpected hiPSC differentiation

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problem. If it is due to disease related gene mutation, other patient samples with different gene mutations may need to be used to generate new hiPSCs.

2. Massive cell death and cell loss occur after several passages, most often observed after 5 to 7 passages after hiPSC generation from somatic cells. A new iPSC colony may need to be selected for culturing again, or a new hiPSC generation should be considered.

3. If the results show incomplete pluripotency markers or abnormality in chromosome morphology (in Karyotyping experiment), this hiPSC colony should be discarded and a new hiPSC colony (from the same hiPSC pool after hiPSC generation) should be selected instead. Additionally, cytogenetic analysis using Karyotyping to check chromosome integrity in original donor cells should be considered. If the original donor cells (somatic cells) show abnormal chromosome integrity, the patient sample should not be used again.

4. Culturing hiPSCs is a tedious process that needs special care, good cell culture skill, and laboratory hygiene technique. If contaminations of bacteria, fungi, or mycoplasma occur, the hiPSCs should be discarded and a new hiPSC vial in stock should be used to restart the culture. We do not recommend attempting cleanup mycoplasma infections in hiPSCs because the “cleanup” is usually incomplete, and the mycoplasma infection will reoccur after a few passages.

Time Considerations:

1. Propagation and cryopreservation of hiPSCs take 10–15 mins. However, once seeded, hiPSC cultures take 4–5 days to reach 70–80% confluence for next passage (Basic Protocol 1). Thawing cells take approximately 1 h: 15–30 sec to thaw a cryovial and 1 h for cell attachment (Basic Protocol 2).

2. It takes approximately 2 days before the hiPSCs are ready for immunostaining. Cell fixing and washing take about 30 min. Cell permeabilization after fixing takes 30 min. Blocking cells takes 1 h at RT. Staining with primary antibodies requires an overnight incubation. Staining with secondary antibodies takes 1.5 h, including 3 cell wash steps. Nuclear staining takes 30 min (Basic Protocol 3).

3. It takes approximately 15–30 min to prepare cell pellets for flow cytometric analysis. Cell fixing and washing take about 20 min. Cell permeabilizing takes 10 min. Antibodies staining takes 1 h. Final cell wash takes 10 min before detection (Alternate Protocol 1).

4. For the teratoma formation assay, hiPSC dissociation, spin down, cell counting, and injection into two immunocompromised mice can usually be done within 1–2 hours. For most cell lines, teratomas can reach the maximum allowed size (usually 2 centimeters in diameter but can vary based on the approved animal protocol) in 6–12 weeks post injection. Euthanizing mice and harvesting teratomas can usually be done in 20 min, but it is likely that the two mice need to be euthanized at different times due to differences in tumor growth rates. Teratomas are usually fixed in 10% Formalin overnight before sending to a CRO

for sectioning and staining, which often takes 2–3 weeks. Examining stained slides (usually three slides from different regions of each tumor) and taking photographs of representative germ layer tissues usually take 15–30 min (Basic Protocol 4).

5. It takes 7 days to prepare positive control samples for the Sendai virus vector assay to confirm elimination of virus vector and transgenes introduced during iPSC generation including 2 days for culturing fibroblasts, and 5 days for iPSC induction. RNA extraction requires 25–30 min. It takes 1.5 to 2 h for PCR sample preparation and amplification, and an additional 30–60 min for detection via gel electrophoresis (Basic Protocol 5).

Other experiments for hiPSC quality control

We described 5 Basic Protocols above in details that are unique for hiPSC culture and characterization. The additional experiments listed in Figure 1b for hiPSC quality control are more general, and usually can be outsourced to core facilities or contract research organization (CRO) using the live hiPSCs (Basic Protocl-1, step 18c) or cryopreserved hiPSCs (Basic Protocol-2, step 12).

1. Karyotyping experiment. This assay is used to check the chromosome integrity in hiPSCs after culturing. If chromosomal abnormality is found, the hiPSCs should be discarded. An example of CRO for G-banding karyotype analysis is the WiCell Research Institute (Madison, WI, USA) which we have used.

2. Short tandem repeat (STR) DNA profile analysis. The STR profiling assay is commonly used to examine if a cell line in culture is same as the original cell line as unexpected cell line contamination and mislabeling can occur during a long-term cell culture. Both the hiPSCs and original somatic cells (e.g., patient fibroblasts) used to generate this hiPSC line are needed for the STR assay to compare if the hiPSCs in culture matches the original donor cells. If the STR profiles of the two cell lines are not match (due to contamination or mislabeling), the hiPSCs must be discarded. We have contracted STR experiments to WiCell Research Institute (Madison, WI, USA).

3. Mycoplasma test. Mycoplasma can be detected and analyzed using an assay kit, and should be routinely tested for cell culture. For example, the MycoAlert Kit (Lonza, Catalog #: LT07-318) can be used by following manufacturer's instruction. The ratio B/A > 1.2 indicates mycoplasma positive; 0.9–1.2 ambiguous result; and <0.9 mycoplasma negative.

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DATA AVAILABILITY STATEMENT:

N/A

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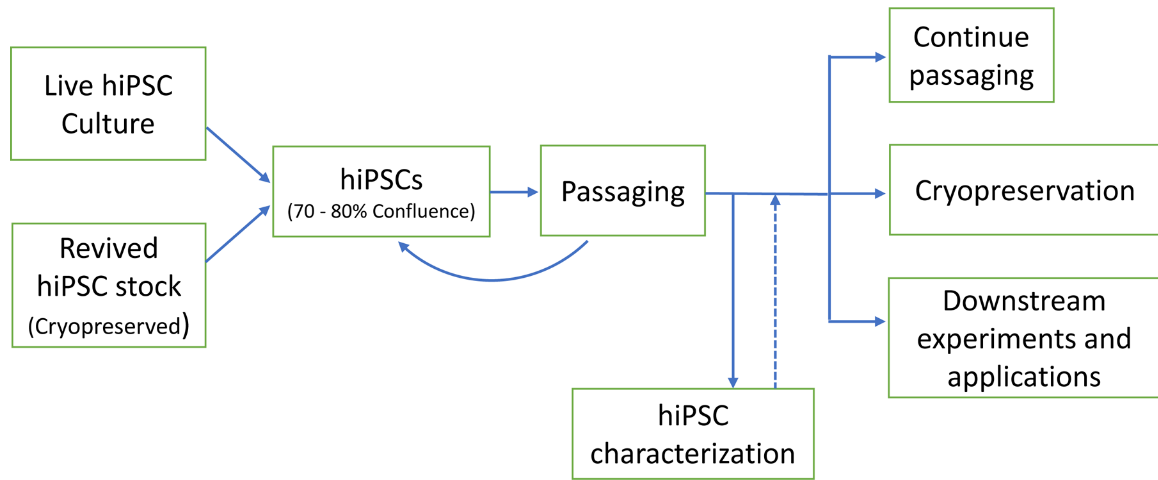

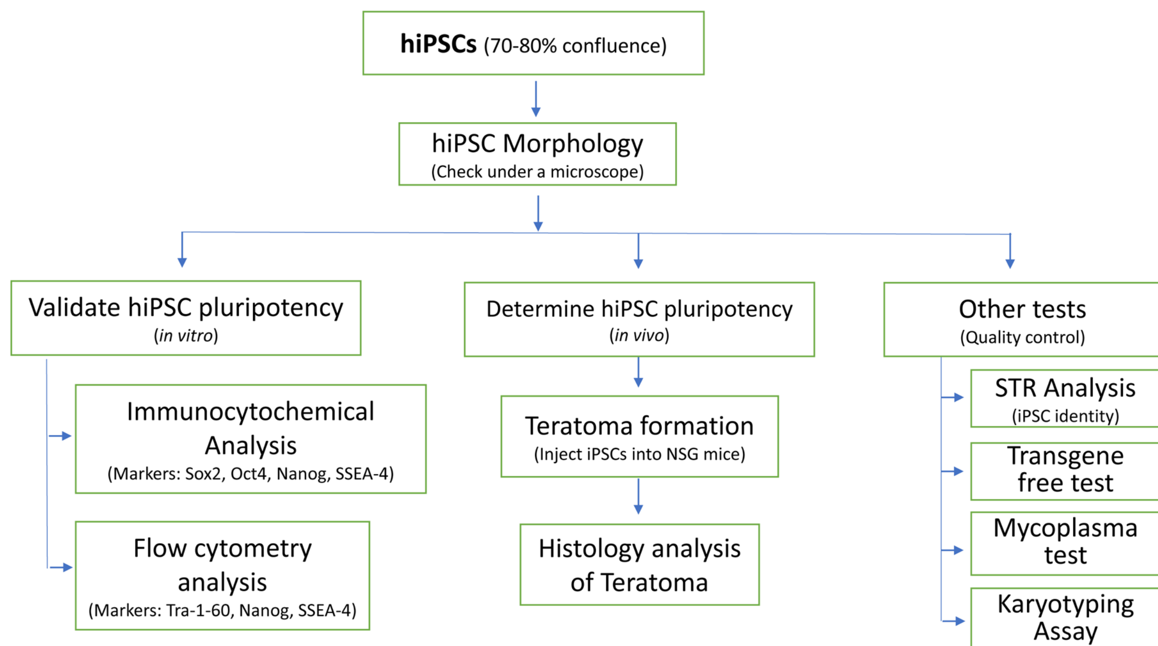

Figure 1.

Flowcharts for hiPSC culture and hiPSC characterization. (A) Human iPSC culture usually starts with live hiPSCs (e.g., after hiPSC generation) by using the Basic Protocol I (Propagation and cryopreservation of hiPSC cultures) for hiPSC passaging and propagation. If a frozen stock vial of hiPSCs is a starting point, the Basic Protocol 2 (Recovery of cryopreserved hiPSCs) should be used followed by the Basic Protocol 1. (B) After a couple rounds of passaging and propagation of hiPSCs, hiPSC characterization is performed to ensure high quality of the hiPSCs including the Basic Protocol 3 (Determination of expression of pluripotency markers via flow cytometry analysis) (and/or the Alternate Protocol 1), Basic Protocol 4 (Pluripotency assessment via in vivo teratoma formation assay), and Basic Protocol 5 (Examination of clearance of Sendai viral vector via reverse

transcription PCR). If good results of hiPSC characterization are obtained as described in these protocols, the next step experiments can be moved forward (the dotted line).

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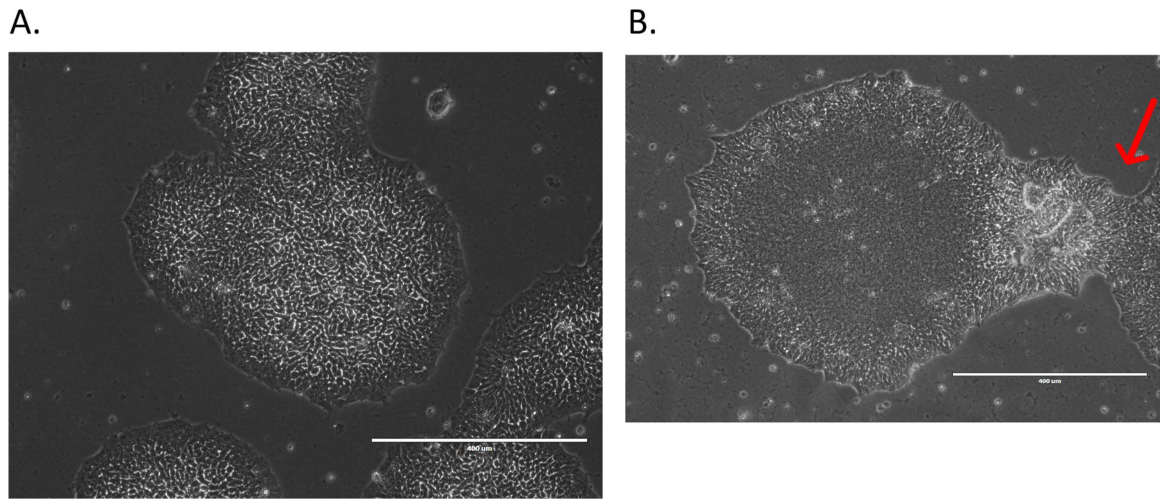

Figure 2.

The morphology of iPSC colonies observed via microscopy. (A) A normal hiPSC colony has distinct border and well-defined edge that consist of cells with a large nucleus (a high ratio of nucleus and cytoplasm). (B) Spontaneous differentiation cells inside a hiPSC colony (arrow)

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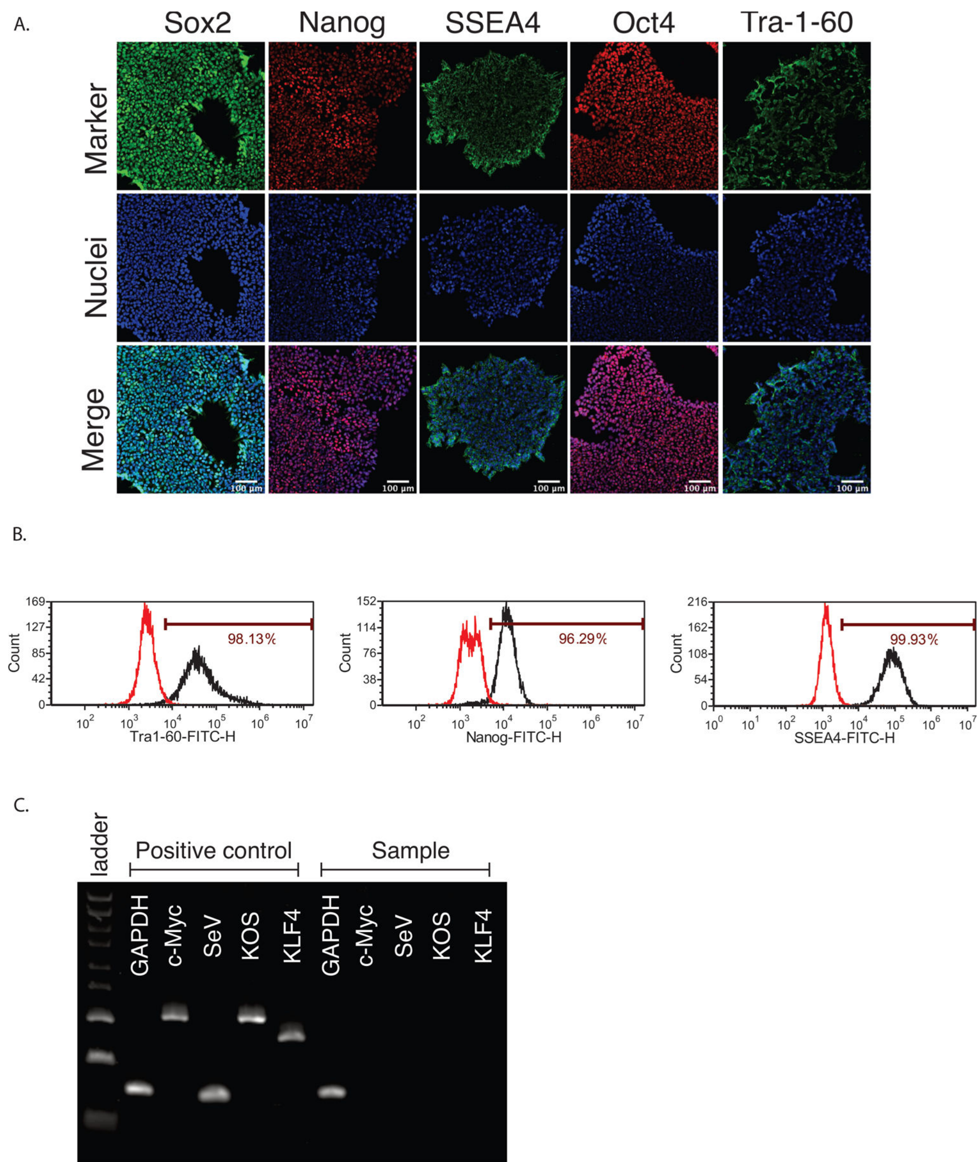

Figure 3.

Characterization of hiPSCs. (A) Immunofluorescence images of hiPSCs stained with pluripotency markers. (B) Flow cytometry analysis of hiPSCs stained with pluripotency markers. (C) RT-PCR results showed that the transgenes (used in hiPSC induction) are not presented in hiPSCs (Sample) after 10 passages. Whereas the transgenes are present in the positive control (freshly transduced fibroblasts).

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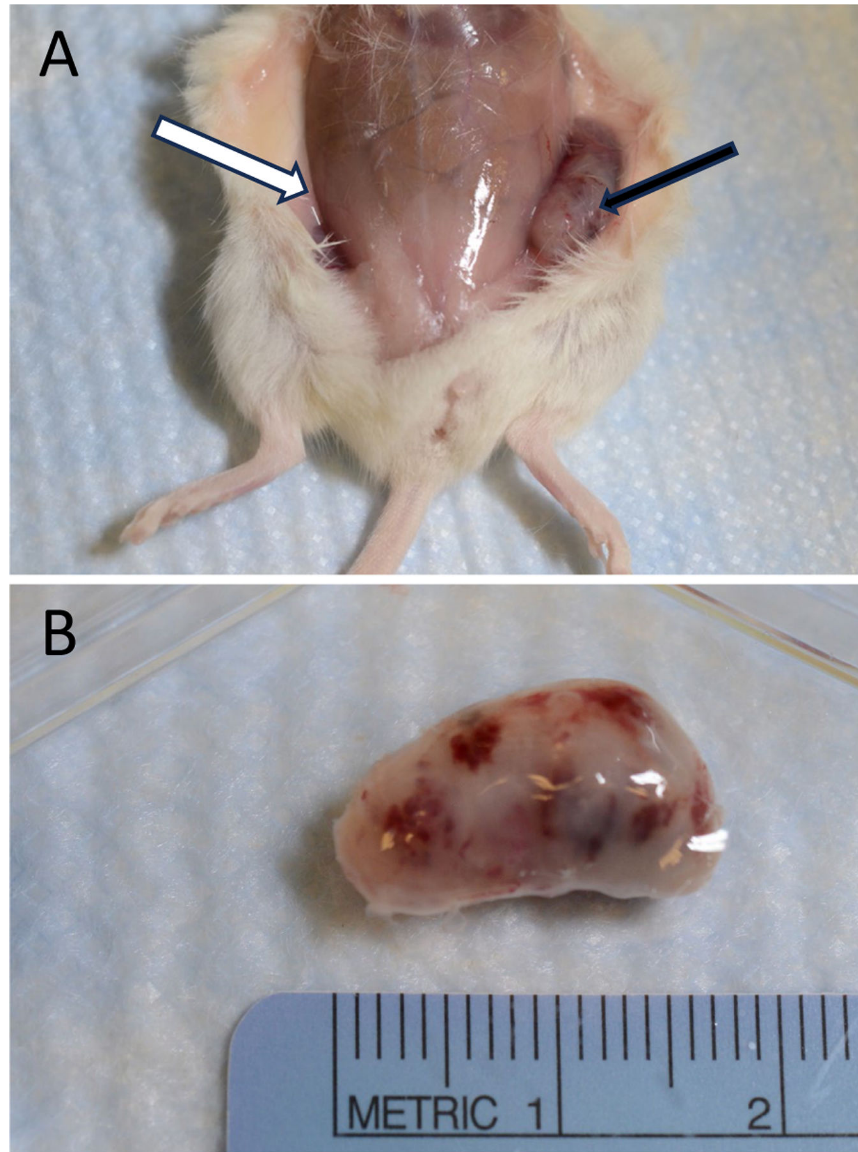

Figure 4.

Gross morphology of a teratoma. A) A subcutaneous teratoma (solid arrow) before removing from an euthanize NSG mouse. The open arrow points to the un-injected control side. B) A microphotograph of a dissected teratoma.

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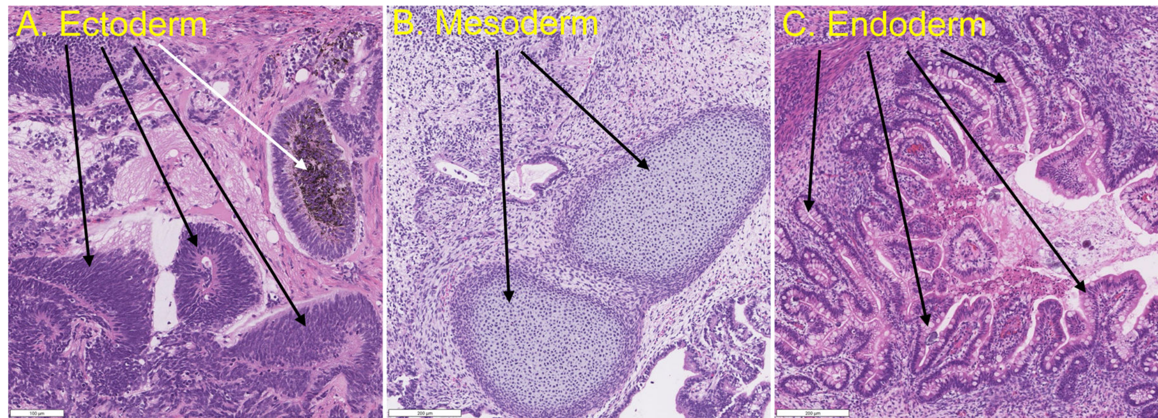

Figure 5.
Typical images representing tissues from the three germ layers in the teratoma formed by hiPSCs. (A) Black arrows point to neural epithelium often referred to as neural rosettes, which are the most typical ectodermal tissue in teratoma assays. Some of the tissues contain pigmented cells (white arrow), resembling retina. (B) The arrows point to cartilage, which is the most easily recognizable tissue derived from the mesoderm. (C) The arrows point to gut tissues, resembling intestine, which is a typical tissue representing endoderm.

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Table 1.

Antibodies and dilutions used for immunocytochemistry.

Usage of Antibody	Antibody host species	Dilution	Company, Cat# and RRID
Primary Antibody	*Mouse anti-SOX2*	*1:50*	*R&D Systems, MAB2018, AB_358009*
	Mouse anti-SSEA4	*1:100*	*Cell Signaling Technology, 4755, AB_1264259*
	Rabbit anti-NANOG	*1:100*	*Cell Signaling Technology, 4903, AB_10559205*
	Rabbit anti-OCT4A	*1:100*	*ThermoFisher Scientific, A13998, AB_2534182*
Secondary Antibody	*Donkey anti-mouse IgG (Alexa Fluor 488)*	*1:400*	*ThermoFisher Scientific, A21202, AB_141607*
	Donkey anti-Rabbit IgG (Alexa Fluor 594)	*1:400*	*ThermoFisher Scientific, A21207, AB_141637*

Note: if different antibodies are used, the antibody dilutions have to be tested and optimized.

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Table 2.

Antibodies and dilutions used for flow cytometry

Antibody	Species	Dilution	Company, Cat# and RRID
Anti-Tra-1-60-DyLight 488	*Mouse IgM*	*1:50*	*ThermoFisher Scientific, MA1–023-D488X, AB_2536700*
Anti-Nanog-Alexa Fluor 488	*Rabbit IgG*	*1:50*	*Millipore, FCABS352A4, AB_10807973*
Anti-SSEA-4-Alexa Fluor 488	*Mouse IgG3*	*1:50*	*ThermoFisher Scientific, 53–8843-41, AB_10597752*
Mouse-IgM-DyLight 488	*Mouse IgM*	*1:50*	*ThermoFisher Scientific, MA1–194-D488, AB_2536969*
Rabbit IgG-Alexa Fluor 488	*Rabbit IgG*	*1:50*	*Cell Signaling, 4340S, AB_10694568*
Mouse IgG3-FITC	*Mouse IgG3*	*1:50*	*ThermoFisher Scientific, 11–4742-42, AB_2043894*

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Table 3.

PCR primers used for Sendai virus vector determination.

	Target	*Forward/Reverse primer (5′–3′)*
Reference Genes	*GAPDH/197bp*	*Fw: GGAGCGAGATCCCTCCAAAAT* *Rv: GGCTGTTGTCATACTTCTCATGG*
SeV specific primers	*SeV/181bp*	*Fw: GGA TCA CTA GGT GAT ATC GAG C* *Rv: ACC AGA CAA GAG TTT AAG AGA TAT GTA TC*
SeV specific primers	*KOS/528bp*	*Fw: ATG CAC CGC TAC GAC GTG AGC GC* *Rv: ACC TTG ACA ATC CTG ATG TGG*
SeV specific primers	*Klf4/410bp*	*Fw: TTC CTG CAT GCC AGA GGA GCC C* *Rv: AAT GTA TCG AAG GTG CTC AA*
SeV specific primers	*C-Myc/523bp*	*Fw: TAA CTG ACT AGC AGG CTT GTC G* *Rv: TCC ACA TAC AGT CCT GGA TGA TGA TG*

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Table 4.

Troubleshooting Guide for iPSC culture and *In vivo* differentiation by teratoma formation

Problem	Possible Cause	Solution
Detectable transgenes in iPSC clone	Transgene(s) randomly integrated into genome	Restarting to generating new iPSC from somatic cells.
Spontaneous differentiation	Differentiated cells were carried over or an unstable clone was selected from the hiPSC generation.	Remove differentiation area prior to passaging or restart from a different hiPSC clone if over 10% of the differentiating population to be found in the culture.
	hiPSCs were kept in Y-27632 dihydrochloride too long (over 24 hours).	Make sure hiPSCs are not cultured with Y-27632 dihydrochloride for a long period of time.
Insufficient cell recovery after thawing	Inappropriate freezing and thawing process.	Optimization steps and culture materials options for the freezing, thawing, and seeding of iPSC on feeder-free, ECM-coated, cell culture plates.
iPSC grow slowly	Cell numbers too low and cell aggregates too small or single cells during cell passaging	While the iPSC grow up to approx. 70–80% confluency, make sure that the cells are still within the log growth phase and split 1:2–1:6 ratio. Avoid harsh pipetting to disrupt the clone in single cells or tiny aggregates during passaging. Normally, a cell aggregate size of 50–200 μm is recommended.
No teratoma growth 12 weeks post injection	The hiPSCs were not cultured at optimal conditions prior to injection, they may have been over confluent, cultured in bad medium (the medium is not freshly made), or were not fed or split on time.	Repeat the injection using the cells in the rapid growing phase (50–70% confluency). .
	The cells were mishandled during harvesting or injection (e.g., too much time between cell harvest and injection), 25 mM HEPES (pH7.4) was omitted from the medium so that the pH of the cell suspension was not maintained properly while outside of the CO_2 incubator, or the high density cell suspension was not kept on ice, which can cause depletion of metabolites due to higher metabolic rate at RT.	Repeat the injection using proper handling methods.
	Too few cells were successfully injected (e.g., cell counting error, or cells leaked out from injection site during cell injection). Use of too thin or too thick a needle (Needles that are too thin can lyse cells, while needles that are too thick can cause leakage).	Repeat the injection using correct number of cells and proper needle size.
	Some hiPSC lines do not form teratomas when injected subcutaneously, which indicates the hiPSCs have lost pluripotency.	Choose a different hiPSC clone. If no alternative clones are available, repeat the injection using more mice with increased number of cells. Try injecting into alternative sites such as intramuscular, kidney capsule, or testicular capsule, which require fewer cells and provide different niches.
Tumors are largely cysts with very little solid tissues, or solid tumors are homogenous with very little well differentiated cell types	The cell line may not be pluripotent or is contaminated with transformed cancerous cells, or certain genes used for reprograming are not fully inactivated, which promotes proliferation and inhibits differentiation.	Choose a different hiPSC clone. If no alternative clones are available, inject more mice (5–6) using properly handled cells in order to confirm whether the cell line is indeed incapable of differentiating into various cell types.
Teratomas contain abundant tissues from certain germ layers but few tissues from other germ layer(s)	Epigenetic memory from donor cells or incomplete hiPSC reprograming; genetic defects that inhibit differentiation into certain cell lineages or promote differentiation into other cell lineages; cell differentiation in teratomas is largely random and sporadic, and the analyzed tumor happens to be abundant for certain tissue types by chance.	Choose different hiPSC clones if they are available. Otherwise, repeat the injection using more mice to confirm the line is biased towards differentiating into a certain tissue type. If confirmed, this cell line could be preferable or non-preferable depending on the purpose of your study.