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Protocol for Directed Differentiation of Human Induced Pluripotent Stem Cells (iPSCs) to a Hepatic Lineage

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Abstract

Directed differentiation is a powerful cell culture technique where developmental pathways are applied to a pluripotent progenitor in order to generate specific terminally differentiated cell populations. Here, we describe a serum-free protocol using growth factors in defined concentrations to derive iPSC-hepatic cells starting from both feeder and feeder-free conditions. The generated iPSC-hepatic cells are developmentally similar to fetal stage hepatocytes, and when generated from patients with genetic mutations such as alpha-1 antitrypsin deficiency recapitulate pathologic changes associated with clinical disease, such as protein misfolding, intracellular retention of misfolded proteins, and elevated levels of ER stress.

Keywords

Induced pluripotent stem cell (iPSC); Directed differentiation; Definitive endoderm; iPSC-hepatic cell

1 Introduction

The ability to maintain embryonic stem cells (ESCs) in an in-vitro culture system has been well described for over 30 years [1]. Their essential characteristics, including the ability to self-renew and differentiate to all three germ layers (ectoderm, endoderm, and mesoderm), resulted in significant interest in their potential therapeutic applications [2]. However, ethical concerns about the destruction of human embryos necessary to generate each genetically distinct ESC line limited progress within the field. In 2006, the field of stem cell biology was dramatically altered by the seminal discovery of Takahashi and Yamanaka that by overexpressing four transcription factors (Oct4, Klf4, Sox2, and cMyc) it was possible to reprogram a terminally differentiated somatic cell back to a pluripotent state [3]. These cells were given the name induced pluripotent stem cells (iPSCs), and as they are created from adult cells such as fibroblasts and monocytes they do not carry the stigma of their ESC

counterparts [4, 5]. Additionally, it was now possible to generate iPSCs from patients with specific genetic and phenotypic characteristics [6].

The introduction of iPSCs has been accompanied by advancements in the ability to derive specific cell lineages from pluripotent progenitors. By providing key growth factors at specific time points, mimicking signals and developmental pathways from the embryo, it is possible to pattern these pluripotent cells into a specific cell fate through a process known as directed differentiation [7]. Multiple differentiated cell types have been successfully derived from iPSCs; however, directed differentiation protocols have been characterized by wide variation in efficiency and maturation potential [8–10]. The ability to derive iPSC-hepatic cells has become well established and represents one of the more efficient protocols as it is possible to achieve >90% iPSC-hepatic cells as marked by AAT+/FOXA1+ cells [10]. To derive iPSC-hepatic cells, pluripotent progenitors are first exposed to the nodal analogue Activin A to derive definitive endoderm as defined by the cellular phenotype ckit+/CXCR4+/FOXA2+/brachyury– [11]. Definitive endoderm is then specified to the hepatic lineage through exposure to high-dose BMP-4 and FGF-2 [12]. Then through treatment with additional growth factors including HGF, gamma secretase inhibitor, and Oncostatin M, the cells undergo maturation [10]. Importantly, after 25 days these cells demonstrate key functional characteristics of fetal hepatocytes and, when derived from patients with genetic disorders (such as alpha-1 antitrypsin deficiency), recapitulate critical components of disease pathogenesis (Fig. 1) [10, 13]. In contrast to primary hepatocytes, which are limited in their ability to proliferate and lose their phenotype in culture, iPSC-hepatic cells provide an unlimited supply of human derived hepatic cells. Additionally, the ability to generate iPSCs from patients with known clinical phenotypes provides the unique opportunity to interrogate specific genetic contributions to liver disease pathogenesis. Finally, there is great interest in whether iPSC-hepatic cells can be used in personalized medicine applications such as drug testing [10].

2 Materials

2.1 Human Cell Culture Components

1. Matrigel. Store at –80 °C (BD Biosciences).
2. Gelatin 0.1% (EMD Millipore).
3. mTeSR1 medium: Maintenance culture medium for hiPSCs, prepare by combining supplied 5× supplement with base medium. Store at 4 °C (Stem Cell Technologies).
4. Dulbecco's Modified Eagle Medium (DMEM): high glucose, L-Glutamine, sodium pyruvate. Store at 4 °C (Thermo Fisher Scientific).
5. Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12). Store at 4 °C (Thermo Fisher Scientific).
6. Iscove's Modified Dulbecco's Media (IMDM). Store at 4 °C (Thermo Fisher Scientific).

7. Dulbecco's Phosphate-Buffered Saline (DPBS): No calcium, no magnesium. Store at 4 °C (Thermo Fisher Scientific).
8. Gentle Cell Dissociation Reagent (Stem Cell Technologies).
9. Collagenase Type IV. Store at -4 °C (Life Technologies).
10. Primocin. Store at -20 °C (Thermo Fisher Scientific).
11. Rho-associated protein kinase (ROCK) inhibitor Y-27632. Store at -80 °C until thawed and then store at 4 °C (Tocris).
12. Defined Fetal Bovine Serum (FBS) heat inactivated. Store at -20 °C (Thermo Fisher Scientific).
13. Knockout Serum. Store at -20 °C (Thermo Fisher Scientific).
14. Non-Essential Amino Acid (NEAA). Store at 4 °C (Life Technologies).
15. Glutamax. Store at 4 °C (Life Technologies).
16. 2-Mercaptoethanol. Store at 4 °C (Life Technologies).

2.2 Differentiation Components

1. Ham's F-12. Store at 4 °C (Cellgro).
2. L-Glutamine- 200 mM. Store at -20 °C (Invitrogen).
3. B27 without Retinoic Acid. Store at -20 °C (Gibco).
4. N2 Supplement. Store at -20 °C (Gibco).
5. Bovine Albumin Fraction V (BSA)—7.5% solution. Store at 4 °C (Invitrogen).
6. 1-Thioglycerol (MTG). Store at -20 °C (Sigma-Aldrich).
7. L-Ascorbic Acid. Store at -20 °C (Sigma-Aldrich).
8. rhBMP4 (*see* Note 1) (R&D Systems).
9. rhFGF-2 (R&D Systems).
10. rhVEGF (R&D Systems).
11. rhEGF (R&D Systems).
12. rhTGF α (R&D Systems).
13. rhHGF (R&D Systems).
14. STEMdiff Definitive Endoderm Kit (Stem Cell Technologies).
15. Dexamethasone. Store at -20 °C (Sigma-Aldrich).
16. Oncostatin-M. Store at -20 °C (Invitrogen).
17. Vitamin-K. Store at -20 °C (Invitrogen).

¹Unless otherwise specified growth factors should be stored at -80 °C and once thawed kept at 4 °C for up to a month.

18. Gamma Secretase Inhibitor (gSI). Store at -20°C (EMD Millipore).

2.3 Antibodies

1. APC anti-human CD117 (c-kit) Antibody. Store at 4°C (BioLegend).
2. Mouse IgG1, APC (c-kit isotype). Store at 4°C (Invitrogen).
3. CXCR4/CD184 Antibody, RPE conjugate. Store at 4°C (Invitrogen).
4. Mouse IgG2a, (R-PE) (CXCR4 isotype). Store at 4°C (Invitrogen).

3 Methods

All cell culture work should be carried out in a biosafety cabinet with laminar flow using a proper sterile technique.

3.1 Hepatic-Directed Differentiation from Feeder-Free Starting Conditions

1. Human iPSC lines should be maintained in culture for at least 1 week prior to using them for differentiation (*see Note 2*). For maintenance, cells should be grown on matrigel-coated tissue culture plates in mTeSR-1 media supplemented with primocin $50\text{ }\mu\text{g/mL}$ (may use pen/strep as well).
2. Cells should be grown until confluent and ready to pass (*see Note 3*).
3. Prepare the necessary number of 6-well plates by coating with matrigel. 1 mg of matrigel should be diluted in 12 mL of cold DMEM/F12. Use 1 mL of matrigel-DMEM/F12 mixture to coat 1 well of a 6-well plate. Plates should sit for 1 h at room temperature or 30 min at 37°C .
4. Aspirate the matrigel and wash once with 2 mL of DMEM/F12. Add 2 mL of mTeSR-1 supplemented with $10\text{ }\mu\text{M}$ ROCK inhibitor.
5. Aspirate media from cells to be passaged and wash with 2 mL PBS. Add 1 mL of Gentle Cell Disassociation (GCD) Reagent to each well and incubate at 37°C for 6 min.
6. Aspirate and add 1 mL of DMEM/F12 and gently wash cells with a P1000 pipette. Transfer to a 15 mL conical (if needed can perform an additional wash step with 1 mL of media to harvest persistently adherent cells).
7. Centrifuge at $300 \times g$ and 4°C for 5 min.
8. Aspirate the supernatant and resuspend in 1 mL of mTeSR-1 supplemented with $10\text{ }\mu\text{M}$ ROCK inhibitor. Gently pipette ten times until a single cell suspension is achieved. Use $10\text{ }\mu\text{L}$ of this suspension to then perform a cell count.

²Although this protocol has been used successfully on multiple distinct wild-type and disease-specific human-iPSC lines, we do observe line-to-line variability in efficiency.

³The number of confluent wells needed is experiment dependent and reflects the number of conditions being tested. Also be sure to save a well of undifferentiated cells to use as a negative control for day 5 FACS analysis.

9. Plate 1×10^6 cells/well of a 6-well plate (*see* Note 4) onto the prepared matrigel plates. The cells should then be moved into a 5% O₂ hypoxic incubator for the remainder of the protocol. This is considered day 0 of the differentiation protocol.

3.2 Hepatic-Directed Differentiation Starting from Feeders

1. For maintenance, iPSCs are grown on MEF-feeder plates in hiPS media (*see* Notes 5 and 6).
2. Cells should be grown until ~70% confluent and ready to pass.
3. Prepare the necessary number of 6-well plates by coating with matrigel, as described above in the feeder-free protocol. Note unlike the feeder-free protocol, cells in this protocol are passed as clumps rather than counted in single-cell suspension. Typically, cells are passed at a split ratio ranging from 1:1 to 1:3.
4. Aspirate hiPS media from cells to be passed and wash cells with 2 mL of DMEM/F12. Add 1 mL of collagenase solution (10 mg/mL) and incubate for 2 min at 37 °C.
5. Aspirate collagenase and wash with 2 mL DMEM/F12. Add 1 mL of hiPS media with 10 µM ROCK inhibitor. Wash and gently scrape 5–10× with a P1000 to detach cells. Note cells will still be in clumps. Transfer to the matrigel-coated well(s) containing an additional 1 mL of hiPS media with 10 µM ROCK inhibitor.
6. Refeed daily with hiPS media for 24–72 h until well is at least 80% confluent. At this point can begin with day 1 of the definitive endoderm step and cells should be moved to a 5% O₂ hypoxic incubator for the remainder of the protocol.

3.3 Definitive Endoderm Days 1–5

1. Prepare day 1 media by mixing 980 µL basal medium with 10 µL of supplement A and 10 µL of supplement B per mL of media using the STEMdiff definitive endoderm kit. We will need to prepare 2 mL of media for each well and supplement with 50 µg/mL of primocin.
2. Aspirate media and wash with 2 mL of DMEM/F12. Feed with day 1 media for 24 h.
3. Prepare days 2–4 media by mixing 990 µL basal medium with 10 µL of supplement B per mL of media and primocin 50 µg/mL. We will need to prepare sufficient volume to refeed with 2 mL of media per well each day.

⁴. We have found plating $1\text{--}1.5 \times 10^6$ cells per well in a 6-well format appears to provide optimal density at day 5. However, as few as 7×10^5 and as many as 2×10^6 cells per well can still provide acceptable results.

⁵. Feeder MEF's need to be mitotically inactivated prior to use with iPSCs. They are available commercially or alternatively can be expanded in house and inactivated with mitomycin-c.

⁶. hiPS media: 200 mL DMEM/F12, 50 mL knockout serum, 2.5 mL Non-Essential Amino Acids, 2.5 mL glutamax, 500 µL 2-mercaptoethanol, primocin 50 µg/mL, and FGF2 5 ng/mL.

4. Aspirate media and wash with DMEM/F12. Feed with 2 mL of days 2–4 media and then refeed every 24 h.
5. Starting at day 5 all media will use the same CSFDM base and should prepare enough for the remainder of the differentiation (*see* Note 7). By day 5 the wells are usually completely confluent (Fig. 2).
6. Prepare days 5–6 media by adding BMP4 10 ng/mL, FGF2 10 ng/mL, and VEGF 10 ng/mL to CSFDM.
7. Prepare new matrigel plates with day 5–6 media supplemented with 10 μ M of ROCK inhibitor.
8. Aspirate old media and wash with 2 mL of DMEM/F12. Add 1 mL of GCD and incubate at 37 °C for 2 min. Note: save 1 well or a portion of a well for FACS analysis to quantify efficiency of differentiation to definitive endoderm (*see* the protocol below).
9. Aspirate GCD and add 1 mL of day 5 media.
10. Gently wash cells by pipetting 5–10 times with P1000 pipette. A confluent well can be passed 1:4 into 4 wells of a new 6-well plate (or 8 wells of a 12-well plate, etc.) (*see* Note 8).

3.4 Day 5 FACS Analysis

1. Collect cells as above (can use either a whole well or a portion of a well if cell numbers are limited) as well as an undifferentiated control well.
2. If using less than an entire well bring volume up to 1 mL. Pipette 10–15 times and use 10 μ L for cell count.
3. Centrifuge cells at $300 \times g$ for 5 min at 4 °C.
4. Aspirate the supernatant and resuspend in 250 μ L of PBS+ (49 mL DPBS and 1 mL FBS) (*see* Note 9).
5. Aliquot 50 μ L into each eppendorf on ice.
6. Add primary antibodies to each tube: 2 μ L of each isotype/ 1×10^6 cells, 5 μ L of ckit/ 1×10^6 cells, 5 μ L of CXCR4/ 1×10^6 cells.
7. Vortex briefly and incubate on ice covered with foil for 30 min.
8. Add 1 mL of PBS+, vortex and centrifuge at $300 \times g$ and 4 °C for 5 min.

⁷-CSFDM media: IMDM 375 mL, Ham's F12 125 mL, B27 without retinoic acid 5 mL, N2 supplement 2.5 mL, 7.5% BSA 3.3 mL, L-glutamine 5 mL, ascorbic acid 5 mL, Diluted MTG (13 μ L MTG diluted in 1 mL IMDM) 1.5 mL, and primocin 50 μ g/mL. After combining the components cover with foil as the media is light sensitive.

⁸-If starting from feeders split no more than 1:3 as there is increased cell death when compared to cells that have already been adapted to feeder-free conditions.

⁹-Can adjust the volume of PBS+ used depending on the number of samples needed. For day 5 definitive endoderm cells will need five conditions: an unstained control, isotypes, single ckit, single CXCR4, and double stain ckit/CXCR4. For undifferentiated controls only need two conditions: unstained control, double stain ckit/CXCR4.

9. Aspirate and resuspend in 400 μ L of FACS buffer pipetting 10–15 $\times$ to resuspend. Filter using 40 μ m filter into FACS tube and keep on ice covered with foil until FACS is performed (*see* Fig. 3 and Note 10).

3.5 iPSC-Hepatic Cells Days 6–25

1. Aspirate media and refeed with 2 mL of day 5–6 media.
2. Prepare day 7–12 media by adding BMP4 50 ng/mL, FGF2 10 ng/mL, VEGF 10 ng/mL, EGF 10 ng/mL, TGF α 20 ng/mL, HGF 100 ng/mL, and dexamethasone 0.1 μ M in CSFDM base media (*see* Note 11).
3. Note before switching between days 7–12, 13–18, and 19–25 medias wash wells once with 2 mL of IMDM to remove any residual growth factors prior to adding the new media. Feed with 2 mL of day 7–12 media. From this point in the protocol cells only need to be refeed every 48 h with fresh media.
4. Prepare day 13–18 media by adding FGF2 10 ng/mL, VEGF 10 ng/mL, EGF 10 ng/mL, HGF 100 ng/mL, oncostatin M 20 ng/mL, vitamin K 6 μ g/mL, dexamethasone 0.1 μ M, gSI 1.5 μ M (*see* Note 12), and DMSO 1% in CSFDM base (*see* Note 13). Refeed with 2 mL every 48 h.
5. Prepare day 19–25 by adding HGF 100 ng/mL, oncostatin M 20 ng/mL, vitamin K 6 μ M, and dexamethasone 0.1 μ M in CSFDM base. Refeed with 2 mL every 48 h.
6. By day 25 the wells should appear completely confluent with the overwhelming majority of cells having the classic hepatocyte polygonal morphology (Fig. 2). If desired supernatants can be collected and analyzed for protein secretion. By this time-point the cells have matured sufficiently that they can be harvested for intracellular FACS analysis (Fig. 1), protein extraction for western blots, RNA extraction (*see* Note 14), or immunostaining.

Acknowledgments

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¹⁰·We routinely see 90+% of cells stain double positive for ckit/CXCR4 (Fig. 3). If endoderm induction is poor (<80%) we would recommend stopping at this point and restarting until a more efficient endodermal induction is achieved. Additionally, it is important that if using multiple different iPSC lines they have similar levels of definitive endoderm or downstream data interpretation could be confounded by the differences in efficiency.

¹¹·Once growth factors are added to the CSFDM base media it lasts not more than 2 weeks and ideally should be used within 7 days.

¹²·gSI is light sensitive and needs to be protected even during storage at –20 °C.

¹³·By day 13 we expect that cells will have started to assume the morphological characteristics of hepatocytes (Fig. 2). Additionally, cells have begun to have visible prominent intracellular inclusions by light microscopy.

¹⁴·For RNA extractions using RLT+ buffer provides low but sufficient yields for qPCR analysis from one well of a 6-well plate. However, if using small well sizes or only a fraction of a well we recommend performing a QIAzol extraction to ensure adequate RNA yields.

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Fig. 1. Intracellular AAT Accumulation in PiZZ Alpha-1 Antitrypsin Deficiency iPSC-Hepatic Cells. Flow cytometry of fixed, permeabilized iPSC-hepatic cells using anti-AAT and anti-FOXA1 antibodies demonstrates a significantly increased level of intracellular AAT protein in PiZZ iPSC-hepatic cells compared to wild-type PiMM cells. This is quantified by the mean fluorescence intensity (MFI) which approximates the amount of stained intracellular AAT protein

Fig. 2. Morphological Changes at Key Time Points during iPSC-hepatic Cell Directed Differentiation. The addition of the modal analogue Activin A to undifferentiated cells helps drive them to definitive endoderm. By day 5, cells no longer form tight distinct colonies but instead have informed a confluent monolayer. Following passaging and treatment with high dose BMP4, HGF, and dexamethasone, the cells begin to assume features typical of hepatocytes, and with further maturation by day 25 have the classic polygonal morphology

Fig. 3. Day 5 Definitive Endoderm Staining. Flow cytometry of day 5 iPSCs using anti-ckit and anti-CXCR4 antibodies demonstrates highly efficient induction of definitive endoderm