3.3 Preparation of MMC-Treated M15 Feeder Plates

1. Take a vial of MMC-M15 cells from −150 °C freezer and put into 37 °C water bath until most cells are thawed.
2. Transfer MMC-M15 cells into a 15 ml tube pre-added with 4 ml EF medium.
3. Spin down at 180 × *g* for 5 min.
4. Resuspend the pellet with EF medium to the concentration at 4.0×10^5 cells/ml.
5. Add 100 µl MMC-M15 cell suspension onto 96-well gelatin-coated plates pre-added with 100 µl EF medium (Section 3.2).
6. Incubate at 37 °C under 5 % CO_2.
7. On the next day, MMC-M15 cells are ready to be used as feeders for human ESCs differentiation.

3.4 Plating and Differentiation of Human ESCs (See Note 5)

1. Remove human ESCs medium.
2. Rinse with PBS.
3. Add 0.25 % trypsin/EDTA and incubate at 37 °C for 5 min.
4. Remove 0.25 % trypsin/EDTA.
5. Add 2 ml EF medium and suspend the cells by pipetting with a P1000 pipet.
6. Add 3 ml EF medium and transfer 5 ml of cells suspension into 15 ml tube.
7. Spin down at 180 × *g* for 5 min.
8. Resuspend the pellet with human ESCs medium supplemented with 10 µM Y27632 to the concentration at 1×10^5 cells/ml.
9. Remove EF medium from the MMC-M15 cells plates (Section 3.3) and add 100 µl fresh human ESCs medium with 10 µM Y27632 into MMC-M15 cells plates.
10. Add 100 µl of cell suspension into MMC-M15 96-well plate pre-added with 100 µl of human ESCs medium.
11. Incubate at 37 °C under 5 % CO_2.
12. On the next day, remove human ESCs medium.
13. Rinse with PBS.
14. Change medium with fresh endoderm differentiation medium 1 supplemented with both Activin and B27 at day 0, 2, 4, 6 from the onset of differentiation.
15. Switch the medium to endoderm differentiation medium 2 supplemented with both Activin and B27 at day 8 from the onset of differentiation and culture cells for 2 days (*see* **Note 8**).

3.5 Plating and Differentiation of Human ESCs (Optional, Feeder-Free System) (See Note 5)

1. Remove human ESCs medium.
2. Rinse with PBS.
3. Add 0.25 % trypsin/EDTA and incubate at 37 °C for 5 min.
4. Remove 0.25 % trypsin/EDTA.
5. Add 2 ml EF medium and suspend the cells by pipetting with a P1000 pipet.
6. Add 3 ml EF medium and transfer 5 ml of cells suspension into 15 ml tube.
7. Spin down at 180 × *g* for 5 min.
8. Resuspend the pellet with human ESCs medium supplemented with 10 μM Y27632 to the concentration at 5×10^5 cells/ml.
9. Remove the solution from matrigel-coated plate and add 100 μl fresh human ESCs medium with 10 μM Y27632 into the plate.
10. Add 100 μl of cell suspension into matrigel-coated plate pre-added with 100 μl of human ESCs medium.
11. Incubate at 37 °C under 5 % CO_2.
12. On the next day, remove human ESCs medium.
13. Rinse with PBS.
14. Change medium with fresh endoderm differentiation medium 1 supplemented with both Activin and B27 at day 0, 2, 4, 6 from the onset of differentiation.
15. Switch the medium to endoderm differentiation medium 2 supplemented with both Activin and B27 at day 8 from the onset of differentiation and culture cells for 2 days (Fig. 1) (*see* **Note 8**).

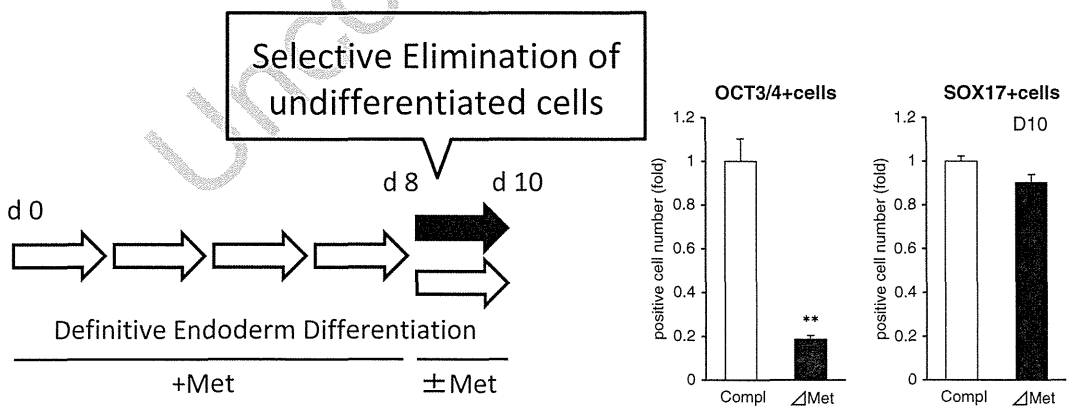

Fig. 1 Human ESCs (khES1) were induced into definitive endoderm through 10-day differentiation, with or without methionine deprivation from differentiation day (d) 8 to d 10. Methionine deprivation resulted in eliminating undifferentiated cells (marked by *OCT3/4* expression) without reducing endoderm cells (marked by *SOX17* expression). Error bars represent SEM ($n = 3$). Significant differences were determined by Student's *t* test; $^{**}p < 0.01$

4 Notes

1. Dissolve three tablets PBS (Sigma, P4417-100TAB) in 600 ml ultrapure water, autoclave, and store at room temperature.
2. Aliquot into 6 ml and store at −20 °C. Avoid freeze and thaw.
3. Dilute 2-mercaptoethanol (Sigma, M7522) to 0.1 M with PBS (i.e. 2-mercaptoethanol (Sigma, M7522) 100 μl/PBS 14.1 ml. Store at 4 °C and use within 1 month.)
4. Dissolve 0.2 g gelatin (Sigma, G9391) in 200 ml ultrapure water. Incubate at room temperature for 1 h, and autoclave, store at room temperature.
5. One day before plating, human ESCs are cultured in human ESCs medium supplemented with 10 μM Y27632. At 80 % confluence, human ESCs are plated.
6. Dissolve 5 mg Y27632 in 1.5 ml distilled water to make 10 mM stock solution. Aliquot into 50 μl and store at −80 °C.
7. Dilute 5 ml Matrigel with 5 ml DMEM (Invitrogen, 11995-075, high Glucose). Aliquot into 100 μl and store at −20 °C. Dilute ten times with DMEM before use.
8. You can combine this procedure with further differentiation (that is, hepatic and pancreatic differentiation, etc.) by continuing cell culture hereafter.

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DOI 10.1007/7651_2014_146

Hepatic Differentiation from Human Ips Cells Using M15 Cells

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Abstract

Here, we describe a procedure of human iPS cells differentiation into the definitive endoderm, further into albumin-expressing and albumin-secreting hepatocyte, using M15, a mesonephros- derived cell line. Approximately 90 % of human iPS cells differentiated into SOX17-positive definitive endoderm then approximately 50 % of cells became albumin-positive cells, and secreted ALB protein. This M15 feeder system for endoderm and hepatic differentiation is a simple and efficient method, and useful for elucidating molecular mechanisms for hepatic fate decision, and could represent an attractive approach for a surrogate cell source for pharmaceutical studies.

Keywords: Hepatic differentiation, Endoderm differentiation, Feeder cells, M15 cells

1 Introduction

Human iPS cells are potential sources of hepatocytes for applications in regenerative medicine and drug development (1). We previously reported a procedure in which ES cells are sequentially induced into the regional specific gut endoderm lineages, such as the pancreas, liver, and intestine, by use of M15, a mesoderm derived cell line (2–4).

M15 is used as a source for signals for in vitro ES differentiation. M15 directs human ES cells to differentiate into the definitive endodermal lineages with the addition of activin and LY294002, a potent PI3 kinase inhibitor, further into the hepatic lineages with the addition of dexamethasone (Dex) and Hepatocyte growth factor (HGF) (2). Approximately 80 % of the human ES cells differentiated into alpha feto protein (AFP)-positive hepatic precursor cells on day 20. On day 40, approximately 9 % of the total cells became Albumin (ALB)-positive hepatocytes and secreted a substantial level of ALB protein (2). Here, we describe an optimized protocol which is more efficient and results in generating a higher portion (85.9 %) of SOX17-positive definitive endoderm by altering the endoderm

differentiation medium (higher concentration of activin, B27 supplement contained RPMI medium) and yielding higher ALB transcription levels (5).

2 Materials

1. M15 cells (ECACC cell no. 95102517).
2. Culture Dish (90-mm, Nunc, 150350) (150-mm, Nunc, 168381).
   (24-well dish, Corning, 3526).
3. PBS (*see* **Note 1**).
4. 0.05 % trypsin/0.53 mM EDTA (Invitrogen, 25300-062).
5. EF medium.

DMEM (Invitrogen, 11995-075)	500 mL
FBS (Hyclone)	58 mL
Penicillin and streptomycin (PS: Nacalai Tesque, 26252-94) (*see* **Note 2**)	5.8 mL
L-Glutamine (Nacalai Tesque, 16948-04) (*see* **Note 2**)	5.8 mL

6. 2× Freeze solution.

EF medium	28 mL
DMSO (Sigma, D2650)	10 mL
FBS (Hyclone)	2 mL

7. Mitomycin C solution.
   Dissolve mitomycin C (2 mg, Sigma, M4287) in 2 mL PBS.
8. Mitomycin C containing medium.

EF medium	200 mL
Mitomycin C solution	2 mL

   The final concentration of mitomycin C will be 10 μg per mL.
9. CTK solution (*see* **Note 3**).

2.5 % Trypsin (Invitrogen, 15090-046)	10 mL
10 mg/mL Collagenase IV (Invitrogen, 17104-019) (*see* **Note 4**)	0.5 mL
Knockout Serum Replacement (KSR,Invitrogen, 10828-028)	20 mL
100 mM $CaCl_2$ (filtrated) (*see* **Note 5**)	1 mL
PBS	59 mL

10. Human iPS medium.

Knockout DMEM/F12 (Sigma-Aldrich)	500 mL
KSR (Invitrogen, 10828-028)	125 mL
PS (Nacalai Tesque, 26252-94) (*see* **Note 2**)	6.25 mL
L-glutamine (Nacalai Tesque, 16948-04) (*see* **Note 2**)	6.25 mL
Nonessential amino acids (NEAA; Invitrogen, 11140-050) (*see* **Note 2**)	6.25 mL
0.1 M β-mercaptoethanol (ME) (*see* **Note 6**)	625 μL

11. Supplements for human iPS medium.

bFGF (Peprotech, 100-18B-2).

Stock solution at 5 μg/mL in 0.1 % (w/v) BSA/PBS. Aliquot into 100 μL and store at −80 °C. Once thawed, keep at 4 °C. Add to human iPS medium at a final concentration of 5 ng/mL.

12. Endoderm Differentiation basal Medium (store at 4 °C).

RPMI 1640 medium (Invitrogen, 11875-093)	500 mL
PS (Nacalai Tesque, 26252-94 (*see* **Note 2**)	5 mL
L-Glutamine (Nacalai Tesque, 16948-04) (*see* **Note 2**)	5 mL
NEAA (Invitrogen, 11140-050) (*see* **Note 2**)	5 mL
0.1 M ME (*see* **Note 6**)	500 μL

13. Supplements for endoderm differentiation Medium (store at 4 °C).

Activin (R&D, 338-AC).

Stock solution at 100 μg/mL in 0.1 % (w/v) BSA/PBS. Aliquot into 100 μL and store at −80 °C. Once thawed, keep at 4 °C. Add to endoderm differentiation medium at a final concentration of 100 ng/mL.

B27 supplement (Invitrogen, 17504-044).

Stock solution at 100 % (50×). Aliquot into 500 μL and store at −20 °C. Once thawed, keep at 4 °C. Add to endoderm differentiation medium at a final concentration of 2 % (v/v, 1×).

14. Hepatic Differentiation basal Medium (store at 4 °C).

DMEM (Invitrogen, 11885-092, low glucose)	500 mL
KSR (Invitrogen, 10828-028)	58 mL
PS (Nacalai Tesque, 26252-94) (*see* **Note 2**)	5.8 mL

(continued)

L-Glutamine (Nacalai Tesque, 16948-04) (*see* **Note 2**)	5.8 mL
NEAA (Invitrogen, 11140-050) (*see* **Note 2**)	5.8 mL
0.1 M ME (*see* **Note 6**)	580 μL
100 mg/mL Glucose (*see* **Note 7**)	5.8 mL

15. Supplements for hepatic differentiation medium.

    Dexamethasone (Dex, Sigma, #D8893).

    Stock solution at 1 mM in EtOH. Aliquot into 100 μL and store at −80 °C. Once thawed, keep at 4 °C. Add to differentiation medium at a final concentration of 1 μM.

    HGF (Peprotech, #100-39).

    Stock solution at 10 μg/mL in 0.1 % (w/v) BSA/PBS. Aliquot into 100 μL and store at −80 °C. Once thawed, keep at 4 °C. Add to differentiation medium at a final concentration of 10 ng/mL.

3 Methods

3.1 Preparation of Mitomycin C Treated M15 Cells (MMC-M15 Cells)

(a) Thawing M15 cells.

1. Prepare 4 mL of EF medium in a 15 mL tube.
2. Remove a vial of frozen M15 stock and put the vial in a 37 °C water bath until most (but not all) cells are thawed.
3. Wipe the vial with ethanol, open the cap, and transfer the cell suspension to a tube prepared in step 1.
4. Centrifuge at 180 × *g* for 5 min and then discard the supernatant.
5. Resuspend the cells with 10 mL of EF medium, and transfer a 90-mm dish, and incubate the cells in a 37 °C 5 % CO_2 incubator.

(b) Passage of M15 cells.

1. When cells are confluent, aspirate the culture medium, wash the cells with PBS, add 0.05 % trypsin/0.53 mM EDTA (1 mL per 90-mm dish, 3 mL per 150-mm dish), and incubate for 5 min at 37 °C, 5 % CO_2.
2. After incubation, add EF medium into the M15 cells dish (4 mL per 90-mm dish, 6 mL per 150-mm dish), suspend the cells by gently pipetting, and transfer the cell suspension to a 15 mL tube or 50-mL tube.
3. Centrifuge the cells at 180 × *g* for 5 min.
4. Discard the supernatant, break the pellet by finger tapping, and resuspend the cells in an appropriate amount of EF medium.

5. Seed cells at 1.5×10^6 cells per 150-mm dish, and incubate at 37 °C, 5 % CO_2 incubator until they are confluent.

(c) Mitomycin C-inactivation of M15 cells.

1. Discard the medium and add mitomycin C containing medium, and incubate for 2 h at 37 °C, 5 % CO_2.
2. After incubation, aspirate all of mitomycin C containing medium off the cells, and wash the cells twice with PBS.
3. Aspirate off PBS, add 3 mL 0.05 % trypsin/0.53 mM EDTA, and incubate for 5 min at 37 °C, 5 % CO_2.
4. Neutralize the trypsin by adding 3 ml EF medium, and break up the cells to a single cell suspension by pipetting up and down. Pool the cells suspension into 50-mL tubes and count the number of cells.
5. Centrifuge at 180 × *g* for 5 min and then discard the supernatant.
6. Resuspend the cells with EF medium to the concentration at 2×10^7 cells per mL.
7. Add equal volume 2× freeze solution, and mix gently.
8. Transfer 1 mL of the cell suspension into cryovial.
9. Put cryovials into a Nalgene controlled-rate freezer box and then put the box into a −80 °C freezer. The next day, transfer the vials of frozen MMC-M15 cells into the −150 °C freezer for long-term storage. When use frozen cells, thaw 1 vial to two 24-well plates.

3.2 Preparation of Gelatin-Coat Plates

1. Transfer enough 0.1 % gelatin solution to cover the bottom of the plates (i.e., 0.5 mL/well for 24-well dish. Let sit at 37 °C for 2 h (*see* **Note 8**).
2. Remove excess gelatin solution, and add 0.25 mL fresh M15 medium into 24-well gelatin-coated plates (*see* **Note 8**).

3.3 Preparation of MMC Treated M15 Feeder Plates

1. Remove a vial of MMC-M15 cells from −150 °C freezer and plunge into 37 °C water bath, agitating the vials until the frozen suspension becomes slurry.
2. Transfer MMC-M15 cells into a 15 mL tube pre-added with 4 mL EF medium.
3. Collect cells by centrifugation at 180 × *g* for 5 min.
4. Resuspend the pellet with EF medium, cell count, and adjust to a final cell density of 4.0×10^5 cells/mL
5. Plate 0.5 mL MMC-M15 cell suspension into 24-well gelatin-coated plates added with EF medium (Section 3.2).
6. Incubate at 37 °C under 5 % CO_2.

7. On the next day, MMC-M15 cells reach confluence and are ready to be used as feeders for human iPS differentiation (*see* **Note 9**).

3.4 Plating of Human iPS Cells (See Note 10)

1. Remove medium from the human iPS cells.
2. Wash with PBS.
3. Add 1 mL CTK solution to the culture dish. Let stand for 6 min at 37 °C and confirm under microscope for detachment of cells.
4. Remove CTK solution from the iPS cells.
5. Add 2 mL human iPS medium and disaggregate iPS clumps into smaller pieces (5–20 cells) by a cell scraper and pipetting by a P1000 pipet.
6. Add 2 mL human iPS medium and collect the cells by centrifugation, at 180 × *g* for 5 min.
7. Resuspend the pellet with 10 mL human iPS medium.
8. Remove M15 medium from the MMC-M15 cells plates (Section 3.3) and add 0.25 mL fresh human iPS medium into MMC-M15 24-well plate (*see* **Note 11**).
9. Add 0.5 mL human iPS cells suspension into MMC-M15 24-well plate.
10. Incubate at 37 °C under 5 % CO_2.
11. Remove medium from the human iPS cells on the next day.
12. Wash with PBS.
13. Change medium with fresh endoderm differentiation medium supplemented with both Activin and B27 on day 1, 3, 5, 7, 9.
14. Change medium with fresh hepatic differentiation medium supplemented with both Dex and HGF from day 10 to 30, every 2 days.

4 Notes

1. Dissolve three tablets PBS (Sigma, P4417-100TAB) in 600 mL ultrapure water, autoclave, and store at room temperature.
2. Aliquot into 5.8 mL and store at −20 °C. Avoid freeze and thaw.
3. Aliquot into 1 mL and store at −20 °C. Avoid freeze and thaw.
4. Dissolve 10 mg of collagenase IV in 1 mL of distilled water, and through with a 0.22 μm pore filter. Aliquot and store at −20 °C.
5. Dissolve 0.11 g of $CaCl_2$ (Nacalai Tesque, 06729-55) in 10 mL of distilled water, and through with a 0.22 μm pore filter.

6. Dilute 2-mercaptoethanol (Sigma, M7522) to 0.1 M with PBS. (i.e., 2-mercaptoethanol (Sigma, M7522) 100 μL/PBS 14.1 mL. Store at 4 °C and use within 1 month.)
7. Dissolve 10 g D-(+)-Glucose (Sigma, G5146-1KG) in 100 mL PBS and filtrate, and store at 4 °C (100 mg/mL).
8. Dissolve 0.2 g gelatin (Sigma, G9391) in 200 mL ultrapure water. Let stand at room temperature for 1 h, and autoclave, store at room temperature.

   We routinely add gelatin solution on the day before plating of M15 cells, incubate until plating. And just before plating, remove gelatin solution and substitute with fresh differentiation medium.
9. If you are in a rush, you can use M15 feeder dishes 2 h after plating. But we routinely plate MMC treated M15 feeders on the previous day.
10. We plate approximately 70 % confluent human iPS cells in 90-mm dish into one 24-well plate.
11. This is necessary to prevent nonuniform platings.

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Biology
DOI 10.1007/7651_2014_138

Hepatic Differentiation from Murine and Human iPS Cells Using Nanofiber Scaffolds

Taiji Yamazoe, Nobuaki Shiraki, and Shoen Kume

Abstract

The induced pluripotent stem (iPS) cells of murine and human are capable to differentiate into any cell type of the body through recapitulating normal development, similarly as the embryonic stem (ES) cells. Lines of evidence support that both ES cells and iPS cells are induced to differentiate in vitro by sequential treatment of humoral cues such as growth factors and chemicals, combined with the use of certain microenvironments including extracellular matrices and scaffolds.

Here, we describe the procedure to potentiate hepatic lineage cells differentiation from murine and human iPS cells, using growth factor cocktails and nanofiber scaffolds. Nanofiber scaffolds have a three-dimensional surface mimicking the fine structures of the basement membrane in vivo, allow the iPS cells to differentiate into the definitive endoderm and mature hepatocyte-like cells more efficiently than the two-dimensional conventional culture plates.

Keywords: Hepatic differentiation, Microenvironment, Extracellular matrices, Nanofiber scaffolds

1 Introduction

The iPS cells and ES cells have the ability to differentiate into any cell type of our body through mimicking normal developmental processes (1, 2). Therefore, these stem cells can serve as an attractive cell source for a large number of cells needed in biomedical research and regenerative therapies.

There are two majorly considerable conditions to culture ES and iPS cells, one is humoral cues in the culture medium and the other is the components of extracellular matrices and scaffolds.

Based on lines of evidence in developmental biology, hepatic differentiation from stem cells has been established (3, 4). This utilized not only the growth factors that are indispensable for liver organogenesis in vivo but also small chemicals that are theoretically expected to evoke intracellular signaling pathways. Activin A, for instance, is a ligand of the TGF-b superfamily and is used to induce endoderm differentiation, and hepatocyte growth factor is used to differentiate cells to adopt differentiation into the hepatic lineages (3, 5).

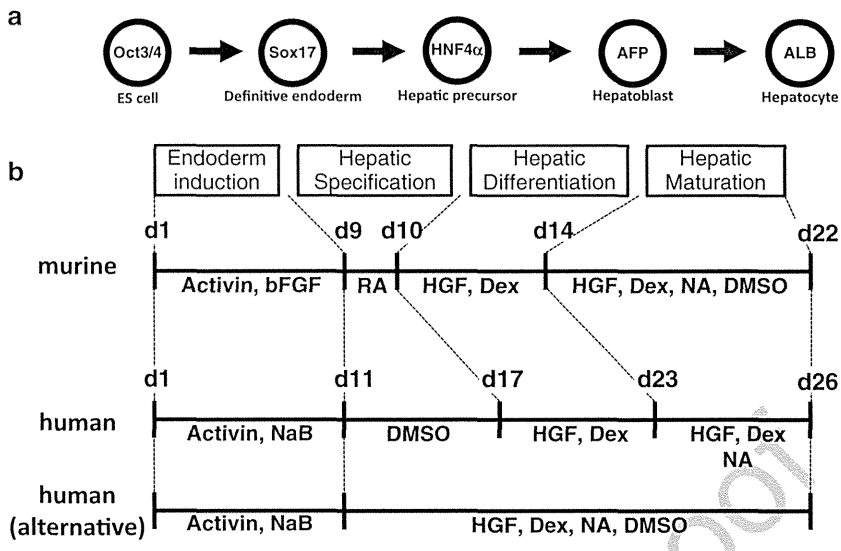

Fig. 1 Scheme for hepatic differentiation program using nanofiber matrices. (**a**) Developmental time course shows a line of differentiating cell profile recapitulating normal developmental process and exhibiting specific marker genes. (**b**) Schedule of medium change shows sequential treatment of specific differentiation cues for each differentiation time windows. *bFGF* basic fibroblast growth factor, *HGF* hepatocyte growth factor, *Dex* dexamethasone, *NA* nicotinamide, *DMSO* dimethyl sulfoxide, *NaB* sodium butylate

Another important factor is the microenvironment including extracellular matrices and scaffolds. We previously reported that culturing ES/iPS cells on a mesonephric cell line, M15, in the presence of specific growth factors, resulted in an efficient induction of endoderm-derived tissues, such as the liver, pancreas, and intestine (6–9). We showed that M15 cells provide basement membrane components, including lama5, on which ES cells could differentiate into regional-specific lineages of the definitive endoderm (10, 11). We then developed an efficient differentiation procedure using synthetic nanofiber matrices for hepatic lineage cells and beta cells (12, 13). The nanofiber matrices show a highly integrated three-dimensional structure that resembles the basement membrane, and provide appropriate guidance cues to modulate cell behavior (14). Here, we demonstrate the nanofiber-based procedure for hepatic differentiation from murine and human iPS or ES cells. This procedure including sequential treatment of growth factors and chemicals to induce endoderm and hepatic lineage cells for 22 days in murine and for 26 days in human (Fig. 1). AU1

2 Materials

2.1 Murine iPS Cell Differentiation

1. Culture Plate(96-well plate, Corning Costar Ultra-Web Synthetic Polyamine Surface, 3873XX1).

2. PBS (*see* **Note 1**).
3. 0.25 % Trypsin-EDTA (Invitrogen, 25200-072).
4. Mouse endoderm differentiation basal medium (store at 4 °C)

DMEM (Invitrogen, 11995-075, high Glucose)	500 mL
AlbuMAX II (Invitrogen, 11021-029) (*see* **Note 2**)	6 mL
Insulin-Transferrin-Selenium-G (Invitrogen, 41400-045)	5 mL
Penicillin/streptomycin (P/S) (Nacalai Tesque, 26252-94)	5 mL
L-Glutamine (Nacalai Tesque, 16948-04)	5 mL
MEM nonessential amino acids solution (NEAA) (Invitrogen, 11140-050)	5 mL
0.1 M 2-mercaptoethanol (*see* **Note 3**)	500 μL

5. Supplements for mouse endoderm differentiation medium

   Activin (R&D, 338-AC): Stock solution at 10 μg/mL in 0.1 % (w/v) BSA/PBS. Aliquot into 100 μL and store at −80 °C. Once thawed, keep at 4 °C. Add to mouse endoderm differentiation basal medium at a final concentration of 10 ng/mL.
   bFGF (Peprotech, 100-18B-2): Stock solution at 5 μg/mL in 0.1 % (w/v) BSA/PBS. Aliquot into 100 μL and store at −80 °C. Once thawed, keep at 4 °C. Add to mouse endoderm differentiation basal medium at a final concentration of 5 ng/mL.
6. Mouse hepatic specification basal medium (store at 4 °C)

RPMI (Invitrogen, 11875-093)	500 mL
B27 supplement (Invitrogen, 17504-044)	10 mL
P/S (Nacalai Tesque, 26252-94)	5 mL
L-Glutamine (Nacalai Tesque, 16948-04)	5 mL
NEAA (Invitrogen, 11140-050)	5 mL
0.1 M 2-mercaptoethanol (*see* **Note 3**)	500 μL

7. Supplements for mouse hepatic specification medium

   Stemolecule™ All-Trans Retinoic Acid (ATRA; Stemgent, #130-095-571): Stock solution at 10 mM in DMSO (Sigma, D2650). Aliquot into 100 μL and store at −80 °C. Once thawed, keep at 4 °C with protection from light. Add to mouse hepatic specification basal medium at a final concentration of 10^{-6} M.

8. Mouse hepatic differentiation and maturation basal medium (store at 4 °C)

DMEM (Invitrogen, 11995-075, high Glucose)	500 mL
KSR (Invitrogen, 10828-028)	58 mL
P/S (Nacalai Tesque, #26252-94)	5.8 mL
L-Glutamine (Nacalai Tesque, #16948-04)	5.8 mL
NEAA (Invitrogen, 11140-050)	5.8 mL
0.1 M 2-mercaptoethanol (*see* **Note 3**)	580 µL

9. Supplements for mouse hepatic differentiation medium

Dexamethasone (Dex) (Sigma, D8893): Stock solution at 1 mM in EtOH. Aliquot into 100 µL and store at −80 °C. Once thawed, keep at 4 °C. Add to mouse hepatic differentiation and maturation basal medium at a final concentration of 1 µM.

HGF (Peprotech, 100-39): Stock solution at 10 µg/mL in 0.1 % (w/v) BSA/PBS. Aliquot into 100 µL and store at −80 °C. Once thawed, keep at 4 °C. Add to mouse hepatic differentiation and maturation basal medium at a final concentration of 10 ng/mL.

10. Supplements for mouse hepatic maturation medium

Nicotinamide (Sigma, N0636-100G): Stock solution at 1 M in PBS. Aliquot into 5 mL and store at −20 °C. Once thawed, keep at 4 °C. Add to mouse hepatic differentiation and maturation basal medium at a final concentration of 1 mM.

Dimethyl Sulfoxide (DMSO) Hybri-Max (Sigma, D2650): Add to mouse hepatic differentiation and maturation basal medium at a final concentration of 1 % (v/v).

11. Mouse iPS plating medium

DMEM (Invitrogen, 11995-075)	500 mL
FBS (Hyclone)	58 mL
P/S (Nacalai Tesque, 26252-94)	5.8 mL
L-Glutamine (Nacalai Tesque, 16948-04)	5.8 mL
NEAA (Invitrogen, 11140-050)	5.8 mL
0.1 M 2-mercaptoethanol (*see* **Note 3**)	580 µL

2.2 Human iPS Cell Differentiation

1. Culture Plate(96-well plate, Corning Costar Ultra-Web Synthetic Polyamine Surface, 3873XX1).
2. PBS (*see* **Note 1**).
3. 0.25 % Trypsin-EDTA (Invitrogen, 25200-072).
4. Matrigel (BD, 354234) (*see* **Note 4**).

5. Y27632 (Wako, 253-00513) (*see* **Note 5**).

6. Human endoderm differentiation basal Medium (store at 4 °C)

RPMI (Invitrogen, 11875-093)	500 mL
B27 supplement (Invitrogen, 17504-044)	10 mL
P/S (Nacalai Tesque, 26252-94)	5 mL
L-Glutamine (Nacalai Tesque, 16948-04)	5 mL
NEAA (Invitrogen, 11140-050)	5 mL
0.1 M 2-mercaptoethanol (*see* **Note 3**)	500 μL

7. Supplements for human endoderm differentiation medium

   Activin(R&D, 338-AC): Stock solution at 100 μg/mL in 0.1 % (w/v) BSA/ PBS. Aliquot into 50 μL and store at −80 °C. Once thawed, keep at 4 °C. Add to human endoderm differentiation basal medium at a final concentration of 100 ng/mL.
   Sodium butyrate (Sigma, B5887-250): Stock solution at 1 M in PBS. Aliquot into 50 μL and store at −20 °C. Once thawed, keep at 4 °C. Add to human endoderm differentiation basal medium at a final concentration of 100 μM.

8. Human hepatic specification basal medium (store at 4 °C)

KnockOut DMEM/F12 (Invitrogen, 12660-012)	500 mL
KSR (Invitrogen, 10828-028)	125 mL
P/S (Nacalai Tesque, 26252-94)	6.5 mL
L-Glutamine (Nacalai Tesque, 16948-04)	6.5 mL
NEAA (Invitrogen, 11140-050)	6.5 mL
0.1 M 2-mercaptoethanol (*see* **Note 3**)	650 μL

9. Supplements for human hepatic specification medium

   Dimethyl Sulfoxide (DMSO) Hybri-Max (Sigma, D2650). Add to human hepatic specification basal medium at a final concentration of 1 % (v/v).

10. Human hepatic differentiation and maturation basal medium (store at 4 °C)

DMEM (Invitrogen, 11995-075, high Glucose)	500 mL
KSR (Invitrogen, 10828-028)	58 mL
P/S (Nacalai Tesque, 26252-94)	5.8 mL
L-Glutamine (Nacalai Tesque, 16948-04)	5.8 mL
NEAA (Invitrogen, 11140-050)	5.8 mL
0.1 M 2-mercaptoethanol (*see* **Note 3**)	580 μL

11. Supplements for human hepatic differentiation medium

Dexamethasone (Dex) (Sigma, D8893): Stock solution at 1 mM in EtOH. Aliquot into 100 μL and store at −80 °C. Once thawed, keep at 4 °C. Add to human hepatic differentiation and maturation basal medium at a final concentration of 1 μM.

HGF (Peprotech, 100-39): Stock solution at 10 μg/mL in 0.1 % (w/v) BSA/PBS. Aliquot into 100 μL and store at −80 °C. Once thawed, keep at 4 °C. Add to human hepatic differentiation and maturation basal medium at a final concentration of 10 ng/mL.

12. Supplements for human hepatic maturation medium

Nicotinamide (Sigma, N0636-100G): Stock solution at 1 M in PBS. Aliquot into 5 mL and store at −20 °C. Once thawed, keep at 4 °C. Add to human hepatic differentiation and maturation basal medium at a final concentration of 0.5 mM.

Dimethyl Sulfoxide (DMSO) Hybri-Max (Sigma, #D2650): Only for 2 step method, add to human hepatic differentiation and maturation basal medium at a final concentration of 0.5 % (v/v).

13. Trypsin stop medium

DMEM (Invitrogen, 11995-075)	500 mL
FBS (Hyclone)	58 mL
P/S (Nacalai Tesque, 26252-94)	5.8 mL
L-Glutamine (Nacalai Tesque, 16948-04)	5.8 mL
NEAA (Invitrogen, 11140-050)	5.8 mL
0.1 M 2-mercaptoethanol (*see* **Note 3**)	580 μL

14. Human iPS cell plating medium

Use appropriate maintenance medium for your ES cells or iPS cells treated with Y27632 at a final concentration of 10 μM.

3 Methods

Carry out all procedure in clean bench and keep the cells in CO_2 incubator with 37 °C, 90 % humidity and 5 % CO_2.

All medium and solution should be use at room temperature or warmed to 37 °C. DO NOT use cold medium at a refrigerator.

Take 200 μL of every differentiation medium for one well of 96-well nanofiber plates.

3.1 Murine iPS Cell: Plating

1. Remove iPS maintenance medium from the cells.
2. Wash with PBS.

3. Add 1 mL 0.25 % trypsin-EDTA solution to the culture dish. Let stand for 5 min at 37 °C and confirm under microscope for detachment of cells.
4. Disperse the cells into a single-cell suspension by pipetting with a P1000 pipet.
5. Add 4 mL mouse iPS plating medium and collect the cells by centrifugation at 180 × *g* for 5 min.
6. Resuspend the pellet with mouse iPS plating medium, cell count, and adjust to a final cell density of 2.5×10^4 cells/mL.
7. Plate 200 µL ES cell suspension into each well of 96-well synthetic nanofiber plate.
8. Incubate at 37 °C under 5 % CO_2 overnight.

3.2 Murine iPS Cell: Differentiation

1. Change medium with fresh mouse endoderm differentiation medium supplemented with both activin and bFGF on day 1, 3, 5, and 7 (*see* **Note 6**).
2. Change medium with fresh mouse hepatic specification medium supplemented with ATRA on day 9, and culture for 24 h.
3. Change medium with fresh mouse hepatic differentiation medium supplemented with both Dex and HGF on day 10 and 12.
4. Change medium with fresh mouse hepatic maturation medium supplemented with all of Dex, HGF, Nicotinamide, and DMSO on day 14 and 16. By changing medium every 2 days it is capable to extend culture.

3.3 Human iPS Cell: Preconditioning

1. In daily changing fresh medium, treat human ES cells or iPS cells with 10 µM Y27632 (ROCK inhibitor) 1 day before plating.

3.4 Human iPS Cell: Plate Preparation

1. Add 50 µL of ten times diluted Matrigel stock solution (final 20 times dilution) onto each well of nanofiber 96-well plate and incubate for more than 3 h at 37 °C under 5 % CO_2.

3.5 Human iPS Cell: Plating

1. Remove medium from the cells.
2. Wash with PBS.
3. Add 1 mL 0.25 % trypsin-EDTA solution to the culture dish. Let stand for 5 min at 37 °C and confirm under microscope for detachment of cells.
4. Disperse the cells into a single-cell suspension by pipetting with a P1000 pipet.
5. Add 4 mL Trypsin stop Medium and collect the cells by centrifugation at 4 °C, 180 × *g* for 5 min.

6. Resuspend the pellet with appropriate ES (iPS) maintenance medium, cell count, and adjust to a final cell density of 5.0×10^5 cells/mL. Add Y27632 into cell suspension to adjust final concentration to 10 μM.
7. Plate 200 μL ES cell suspension into each well of 96-well synthetic nanofiber plate.
8. Incubate at 37 °C under 5 % CO_2 overnight.

3.6 Human iPS Cell: Differentiation

Change medium every 2 days in the indicated period with specified medium.

1. Change medium with fresh human endoderm differentiation medium supplemented with both activin and sodium butyrate on day 1, 3, 5, 7, and 9 (*see* **Note 6**).
2. Change medium with fresh human hepatic specification medium supplemented with DMSO on day 11, 13, and 15.
3. Change medium with fresh human hepatic differentiation medium supplemented with both Dex and HGF on day 17, 19, and 21.
4. Change medium with fresh human hepatic maturation medium supplemented with all of Dex, HGF, Nicotinamide on day 23 and 25. By changing medium every 2 days it is capable to extend culture.

3.7 Human iPS Cell: Differentiation (2 Step Methods, Alternative for Responsive Cell Line)

1. Change medium with fresh human endoderm differentiation medium supplemented with both activin and sodium butyrate on day 1, 3, 5, 7, and 9.
2. Change medium with fresh human hepatic differentiation medium supplemented with all of Dex, HGF, Nicotinamide, and DMSO every 2 days from day 11 to 25. By changing medium every 2 days it is capable to extend culture.

4 Notes

1. Dissolve three tablets of PBS (SIGMA, #P4417-100TAB) in 600 mL ultrapure water, autoclave, and store at room temperature.
2. Dissolve 25 g AlbuMAX II in 125 mL ultrapure water with stirring. Sterilize them with filtration (Millipore, SCGPS05RE). Aliquot into 2 mL and store at −20 °C.
3. Dilute 2-mercaptoethanol (Sigma, M7522) to 0.1 M with PBS (i.e., 2-mercaptoethanol 100 μL/PBS 14.1 mL). Store at 4 °C and use within 1 month.

4. Dilute 5 mL Matrigel with 5 mL DMEM (Invitrogen, 11995-075, high Glucose). Aliquot into 100 μL and store at −20 °C. Dilute 10 times with DMEM before use.
5. Dissolve 5 mg Y27632 in 1.5 mL ultrapure water to make 10 mM stock solution. Aliquot into 50 μL and store at −80 °C.
6. While changing medium for the first time, as cells are attached on the nanofiber surface weakly, dispense the fresh medium gently, not blowing them up.

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