Pancreatic Beta Cell Differentiation From Human Pluripotent Stem Cells

Lina Sui,[1,2] Rudolph L. Leibel,[1] and Dieter Egli[1]

[1]Naomi Berrie Diabetes Center & Department of Pediatrics, College of Physicians and Surgeons, Columbia University Medical Center, New York
[2]Corresponding author: *ls3178@cumc.columbia.edu*

Insulin-expressing beta cells are crucial for the maintenance of systemic glucose homeostasis. Elucidation of the molecular and cellular mechanisms of beta cell development, expansion, survival, and function are required for full understanding of the molecular pathogenesis of diabetes. However, access to human beta cells for such studies is limited by virtue of the logistics of acquisition, prior medical status of donor, and imperfect culture systems for maintaining beta cell identity and function after isolation from human pancreas. Here, a technique for generation of beta cells from human pluripotent stem cells (hPSCs) by modification of key signaling pathways during islet development is described. Up to 70% C-peptide–positive beta cells can be obtained from endodermal anlagen after 27 days of differentiation with specific growth factors and small molecules. Although 50% of them are monohormonal C-peptide–positive cells and have molecular and cellular characteristics consistent with human beta cells in the Islets of Langerhans, a sub-population co-expressing other endocrine markers are also generated, indicating the immaturity of these cells. © 2018 by John Wiley & Sons, Inc.

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INTRODUCTION

The ability to generate (and genetically manipulate) essentially unlimited healthy beta cells from human pluripotent stem cells (hPSCs) enables many aspects of the analysis of the etiology of beta cell dysfunction and, ultimately, the provision of cells for therapeutic transplantation. Since the derivation of the first human embryonic stem cell line (Thomson et al., 1998), many advances have been made in the *in vitro* differentiation of human stem cells into pancreatic beta cells (D'Amour et al., 2006; Mfopou, Chen, Mateizel, Sermon, & Bouwens, 2010; Pagliuca et al., 2014; Rezania et al., 2014). Based on these studies, an efficient and reproducible protocol to generate beta cells from hPSCs—including human-induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs) (Sui et al., 2018)—has been developed. This protocol is based on six key cellular induction steps in hPSCs. (1) A commercial definitive endoderm differentiation kit is used to activate Activin A and Wnt 3a signaling pathways to give rise to 95% SOX17 and FOXA2-positive definitive endoderm cells. (2) Further induction of definitive endoderm cells to primitive gut tube with FGF7 is performed as described in D'Amour et al. (2006). (3) The posterior foregut tube is induced by inhibition of sonic hedgehog and the BMP4 signaling pathway and activation of the retinoic acid pathway (D'Amour et al., 2006;

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Mfopou & Bouwens, 2008; Mfopou et al., 2010). (4) Further commit cells into pancreatic progenitors expressing PDX1 and NKX6.1 by activation of protein kinase C pathway with EGF (Nostro et al., 2015; Sui, Geens, Sermon, Bouwens, & Mfopou, 2013). (5) Perform pancreatic endocrine lineage commitment by addition of thyroid hormone and upregulation of NGN3 expression through inhibition of Notch signaling together with blockage of the TGF beta signaling pathway with ALK5 inhibitor (Rezania et al., 2014). (6) Endocrine progenitors further mature in medium with addition of FBS.

Basic Protocol 1 describes the preparation of hPSCs before initiation of beta cell induction. Basic Protocol 2 details beta cell differentiation from definitive endoderm to beta cells. Quality control measures for various steps in these protocols are provided in the Support Protocol.

BASIC PROTOCOL 1

PREPARATION OF hESCs OR hiPSCs FOR BETA CELL DIFFERENTIATION

hiPSCs are reprogrammed from human somatic cells with pluripotent transcription factors (Mandal & Rossi, 2013). hESCs are derived from the inner cell mass of human blastocysts (Thomson et al., 1998). Before initiation of an experiment, the first step is to ask a question and plan the appropriate scale of the experiment that is necessary to address such question. hPSCs should be cultured for the purpose of a specific experiment. Prepare stock vials of frozen cells that have a normal karyotype. Thaw one vial of cells and expand cells according to the experiment scale. Cells should also be prepared along with the experiment in a small scale for quality control during differentiation (see Support Protocol).

Materials

Geltrex LDEV-Free hESC-qualified Reduced Growth Factor Basement Membrane Matrix (Thermo Fisher Scientific, cat. no. A1413302)
DMEM/F12 medium (Thermo Fisher Scientific, cat. no. 11320-082)
hPSCs: hES or iPS cell line
StemFlex medium (Thermo Fisher Scientific, cat. no. A3349401)
Y-27632, ROCK inhibitor (Selleckchem, cat. no. S1049)
TrypLE Express Enzyme (ThermoFisher, cat. no. 12605-036)

4- and 6-well cell culture plates (Thermo Scientific, cat. no. 14-832-11)
37°C, 5% CO_2 humidified incubator
37°C water bath
15- and 50-ml conical tubes
Centrifuge
Levy counting chamber (VWR Scientific, cat. no. 15170-208)
Inverted microscope

Prepare plates

1. Thaw Geltrex overnight at 4°C, and dilute Geltrex 1:100 in cold DMEM/F12 medium.
2. Distribute diluted Geltrex immediately into each well of 6-well plates (1 ml/well).

   Prepare two 4-well plates and coat one well of each 4-well plate with Geltrex (250 μl/well), included as quality control during differentiation as described in Support Protocol.

3. Place Geltrex-coated plates 1 hr in a 37°C, 5% CO_2 humidified incubator before seeding cells.

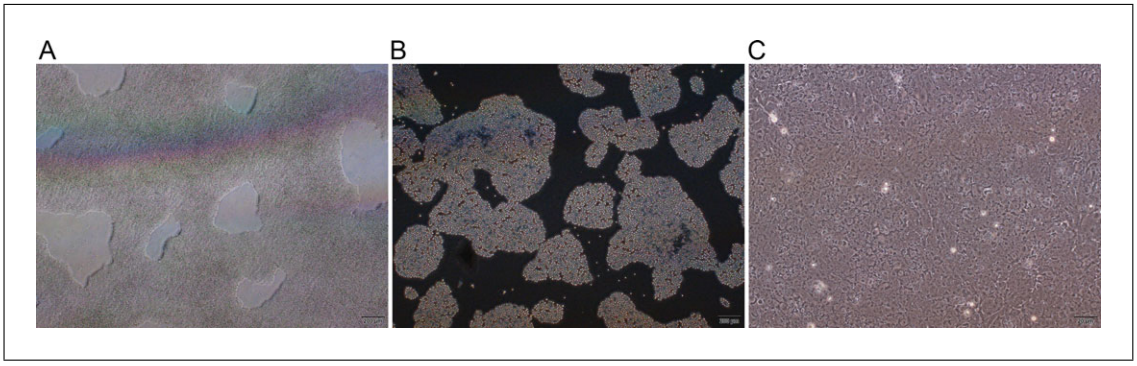

Figure 1 Preparation of human pluripotent stem cells for differentiation. (**A**) hPSCs grown on Geltrex-coated plate in StemFlex medium. Scale bar: 200 μm. (**B**) hPSCs grown on Geltrex-coated plate after TrypLE dissociation. Scale bar: 200 μm. (**C**) hPSCs grown on Geltrex-coated plate in StemFlex medium at 24 hr after splitting and before differentiation. Scale bar: 20 μm.

Prepare cells

4. Thaw hPSCs at 37°C and transfer to a 15-ml conical tube with 5 ml StemFlex medium.
5. Centrifuge cells 5 min at 200 × *g*, room temperature and aspirate supernatant.
6. Resuspend cells in 2 ml StemFlex medium containing 10 μM ROCK inhibitor.
7. Transfer 2 ml StemFlex medium with cells into one well of a Geltrex-coated 6-well plate.

Expand cells

8. Maintain and expand hES or iPS cells using StemFlex medium until hES or iPS colonies are 80% to 90% confluent (Fig. 1A).

   If differentiation experiment is not planned, passage cells with TrypLE Express every 5 to 6 days once cells reach 80% confluence and split at a ratio of 1:10 in the presence of ROCK inhibitor. If differentiation of hPSCs is planned, proceed with the following steps.

9. Detach hES or iPS cells using TrypLE express enzyme (0.5 ml/well) for 2 min at room temperature.

   Check cells under the microscope after adding TrypLE, and stop digestion once cells are dissociated from each other and become round and bright but not yet detached from the plate (Fig. 1B).

10. Aspirate TrypLE express enzyme from the plate, and add 2 ml StemFlex medium containing 10 μM ROCK inhibitor to each well, pipet up and down using a 1000-μl pipet and tip to detach all cells from plate.

    Pipetting up and down six times is sufficient for detachment and to obtain a single-cell suspension. Incubate cells longer with TyrpLE express enzyme, if detachment is insufficient.

11. Transfer cell suspension to a 15-ml conical tube, and wash wells once with 2 ml StemFlex medium containing 10 μM ROCK inhibitor to collect remaining cells.
12. Mix cell suspension by pipetting up and down two times, and load 10 μl into a counting chamber and count cells using an inverted microscope. Do not centrifuge.
13. Calculate the number of cells in 4 ml medium collected from the well. Adjust volume of medium to give ~0.6×10^5 cells/ml.
14. Remove Geltrex-coated plates from the 37°C incubator, and aspirate the Geltrex from each well.

15. Add ~1.2 × 10^6 cells to each well of 6-well plates (2 ml) and ~0.3 × 10^5 cells to one well of each 4-well plate (0.5 ml), and culture 16 to 24 hr in a 37°C, 5% CO_2 incubator.

    The seeded cells are ready for differentiation when they reach 95% to 100% confluency (Fig. 1C).

    Most cell lines will reach 95% to 100% confluency in 16 hr. For cell lines with slow proliferation rates, they may need to be maintained in the same medium containing ROCK inhibitor for 24 hr to reach desired confluency before changing to differentiation medium.

BASIC PROTOCOL 2

STEP-WISE DIFFERENTIATION OF PANCREATIC BETA CELLS FROM hESCs AND hiPSCs

After 95% to 100% confluency is achieved, beta cell induction can be initiated by using combinations of growth factors and small molecules. The differentiation steps are summarized in Figure 2A (i.e., definitive endoderm induction, primitive gut tube

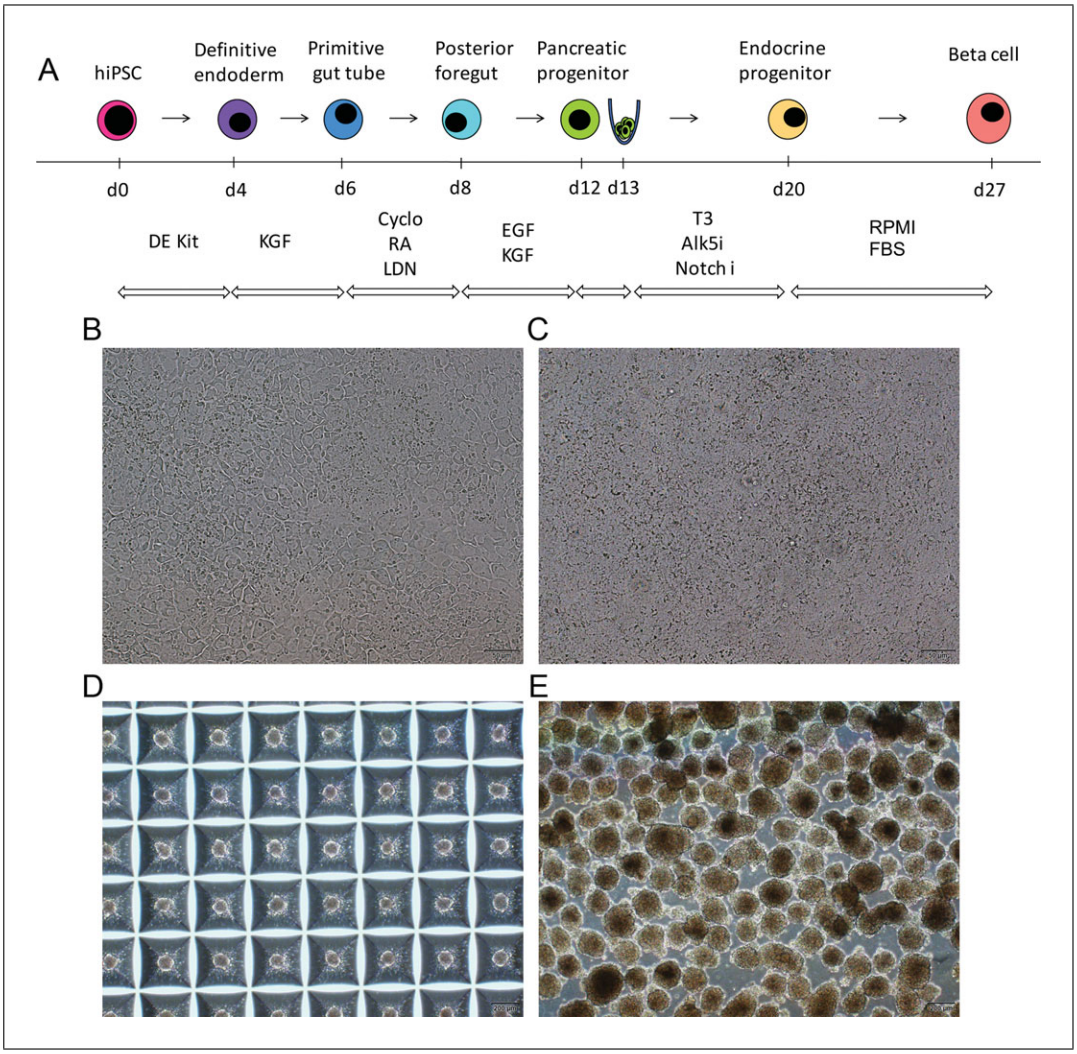

Figure 2 Differentiation of hPSCs towards pancreatic lineage. (**A**) Schematic of differentiation process from hPSC to beta cells. Cells are at (**B**) definitive endoderm stage, (**C**) pancreatic progenitor stage. Scale bar: 50 μm. (**D**) Clusters are formed in AggreWells at 1 day after dissociation of cells at pancreatic progenitor stage. Scale bar: 200 μm. (**E**) Beta cell clusters in low-attachment, 6-well plate. Scale bar: 200 μm.

induction, posterior foregut induction, pancreatic progenitor induction, pancreatic endocrine progenitor induction, and pancreatic beta cell induction). At the pancreatic progenitor stage, cells will be cultured as organoids using AggreWell 400 6-well plates to promote cell-to-cell interaction.

Materials

STEMdiff™ Definitive Endoderm Differentiation Kit containing definitive endoderm basal medium, supplement A, and supplement B (STEMCELL Technologies, cat. no. 05110)
Plated cells (see Basic Protocol 1, step 15)
Washing medium (see recipe)
Primitive gut tube stage medium (see recipe)
Posterior foregut stage medium (see recipe)
Pancreatic progenitor stage medium (see recipe)
Cluster medium (see recipe)
AggreWell Rinsing Solution (STEMCELL Technologies, cat. no. 07010)
TrypLE Express Enzyme (ThermoFisher, cat. no. 12605-036)
DMEM plus GlutaMAX (Life Technology, cat. no. 10569-044)
Pancreatic endocrine progenitor stage medium (see recipe)
Pancreatic beta cell stage medium (see recipe)

37°C water bath
37°C, 5% CO_2 humidified incubator
AggreWell 400 6-well plate (STEMCELL Technologies, cat. no. 34425)
Low-attachment 96-well plates (Corning, cat. no. 7007), optional
Centrifuge with plate attachments
Inverted microscope
15- and 50-ml conical tubes
Low-attachment 6-well plates (Thermo Fisher Scientific, cat. no. 07-200-601)

Induce definitive endoderm (day 0 to day 1)

1. Thaw STEMdiff™ Definitive Endoderm Differentiation Kit.

   Definitive endoderm basal medium can be thawed overnight at 4°C or 30 min in a 37°C water bath. Supplement A and supplement B are thawed at room temperature when the definitive endoderm stage medium is prepared.

2. Prepare definitive endoderm medium for day 1 by mixing both supplement A and supplement B with definitive endoderm basal medium in a 1:100 ratio.

3. Aspirate StemFlex medium from wells containing cells ready for differentiation (see Basic Protocol 1), and wash cells one time by adding 2 ml washing medium per well and gently rocking plate forwards and backwards five times.

4. Replace washing medium with the first day definitive endoderm medium (2 ml/well for 6-well plates and 0.5 ml/well for 4-well plates), and incubate 24 hr in a 37°C, 5% CO_2 humidified incubator.

Days 1 through 2.5

5. Prepare the second day definitive endoderm medium by mixing supplement B with definitive endoderm basal medium in a 1:100 ratio.

   Prepare double the amount of the second day definitive endoderm medium and store at 4°C for the following step.

6. Replace the first day definitive endoderm medium with the second day definitive endoderm medium (2 ml/well for 6-well plates and 0.5 ml/well for 4-well plates), and incubate 36 hr at 37°C.

   A large amount of cell death is expected at this stage, but the plate should remain covered with a monolayer of cells.

Days 2.5 through 4

7. Remove the old medium, and refresh cells with the new second day definitive endoderm medium prepared in step 6 (2 ml/well for 6-well plates and 0.5 ml/well for 4-well plates), and incubate 36 hr in a 37°C, 5% CO_2 humidified incubator.

   A large amount of cell death is expected at this stage, but the plate is still covered with a monolayer of cells (Fig. 2B).

8. Fix cells in 1 well of a 4-well plate for analysis (see Support Protocol).

Induce primitive gut tube (days 4 through 6)

9. Prepare primitive gut tube stage medium.

10. Wash cells with washing medium as described in step 3.

11. Replace washing medium with primitive gut tube stage medium (2 ml/well for 6-well plates and 0.5 ml/well for a 4-well plate), and incubate 48 hr at 37°C.

Induce posterior foregut stage (days 6 through 8)

12. Prepare posterior foregut stage medium.

13. Replace medium with posterior foregut stage medium (2 ml/well for 6-well plates and 0.5 ml/well for a 4-well plate), and incubate 48 hr at 37°C.

Induce pancreatic progenitor stage (days 8 through 12)

14. Prepare pancreatic progenitor stage medium.

    Prepare double the amount of pancreatic progenitor stage medium and store at 4°C for the following step.

15. Replace the posterior foregut stage medium with pancreatic progenitor stage medium (2 ml/well for the 6-well plates and 0.5 ml/well for the 4-well plate), and incubate 48 hr at 37°C.

16. Replace medium with new fresh medium prepared in step 13, and incubate an additional 48 hr at 37°C.

    At day 12, the cells should be compacted and form a monolayer (Fig. 2C).

17. Fix cells in 1 well of a 4-well plate for analysis (see Support Protocol).

Prepare 3-D cell clusters (days 12 and 13)

18. Calculate the amount of cluster medium and the number of wells in a AggreWell 400 6-well plate necessary according to the experiment scale.

    For two wells of a 6-well plate, 5 ml cluster medium and one well of the AggreWell 400 6-well plate are used. Prepare 50 ml cluster medium and one full AggreWell 400 6-well plate. If not used immediately, cluster medium can be stored 3 days at 4°C or at least 1 month at −20°C. AggreWell plates containing AggreWell Rinsing Solution can be placed in the cell incubator until used. If only one well of 6-well plate is planned for differentiation or large size clusters are expected, low-attachment 96-well plates can be used in place of AggreWell 400 6-well plates.

19. Pre-treat the AggreWell 400 6-well plate by adding 2 ml AggreWell Rinsing Solution into each well and centrifuge the plate 5 min at 1300 × *g*, room temperature.

20. Prepare cluster medium.

21. Detach cells at pancreatic progenitor stage by incubating with TrypLE Express Enzyme (0.5 ml/well) for 2 min at room temperature.

    Check cells under microscope after adding TrypLE to ensure that over-digestion does not occur. Stop digestion process once cells detach from each other, and are round and bright, not detached from the plate.

22. Aspirate dissociation enzyme from plate, add 1 ml cluster medium to each well, pipet up and down six times using a 1000-µl pipet and tip to detach all cells from the plate.

    Pipetting up and down six times is sufficient for cell detachment and creates a single-cell suspension. Incubate cells longer with TyrpLE express enzyme, if cells are not detached.

23. Transfer cell suspension to a 50-ml conical tube, and wash the well one time with 1 ml cluster medium to ensure all cells are collected and transfer to the 50-ml conical tube.

    For two wells of a 6-well plate, 4 ml cell suspension is expected.

24. Aspirate AggreWell Rinsing Solution from AggreWell 400 6-well plate after centrifugation from step 16, and rinse well with 1 ml DMEM plus GlutaMAX.

25. Aspirate DMEM plus GlutaMAX medium, transfer 4 ml cell suspension to the well of AggreWell 400 6-well plate, and incubate plate 24 hr at 37°C.

    The 3-D clusters will form in the AggreWell (Fig. 2D).

    Do not shake the plate after seeding cells, move slowly and steady during transportation of the plate from cell culture hood to the incubator.

Induce pancreatic endocrine progenitor stage (days 13 through 20)

26. Calculate the amount of pancreatic endocrine progenitor stage medium and the number of low-attachment 6-well plates will be necessary.

    For one well of an AggreWell 400 6-well plate, 7 ml pancreatic endocrine progenitor stage medium and three wells of a low-attachment 6-well plate are required. Prepare 50 ml pancreatic endocrine progenitor stage medium. If medium is not used immediately, pancreatic endocrine progenitor stage medium can be stored 3 days at 4°C or at least 1 month at −20°C.

27. Prepare pancreatic endocrine progenitor stage medium.

28. Resuspend cell clusters in the AggreWell 400 6-well plate with the medium in the wells, and then collect into a 50-ml conical tube.

29. Rinse well with 1 ml pancreatic endocrine progenitor stage medium and transfer into the same 50-ml conical tube.

30. Pellet cell clusters 5 min in the 50-ml conical tube by gravity.

31. Aspirate supernatant from the 50-ml conical tube and add 6 ml pancreatic endocrine progenitor stage medium to cells.

    To ensure the yield of clusters, leave a small amount of cluster medium in the tube.

32. Resuspend clusters by gently pipetting up and down two times, and transfer suspension into a low-attachment 6-well plate (2 ml/well) (Fig. 2E).

33. Shake plate forwards and backwards and side to side before placing into a 37°C, 5% CO_2 incubator.

34. Change medium every other day by collecting clusters from all wells into a 50-ml conical tube, pellet cells by gravity for 5 min, replace old medium with new medium, and transfer cluster suspension back into the same low-attachment 6-well plate.

Induce pancreatic beta cell stage (days 20 through 27)

35. Prepare pancreatic beta cell stage medium.

36. Change medium every other day as described in step 34.

37. Collect five to ten clusters for analysis (see Support Protocol).

SUPPORT PROTOCOL

CONFIRMATION OF CELL TYPES AT DIFFERENT STAGES DURING DIFFERENTIATION

Cells at different differentiation stages express lineage-specific markers. During the differentiation, immunocytochemistry is used to confirm the derivation of definitive endoderm cells, pancreatic progenitors, and beta cells. To use costly antibodies efficiently during definitive endoderm and pancreatic progenitor stages, a small-scale experiment, along with cells differentiated in 6-well plates and 4-well plates, is performed. At the pancreatic beta cell stage, clusters are collected for cryosectioning.

Additional Materials *(also see Basic Protocols 1 and 2)*

PBS (see recipe)
4% paraformaldehyde (PFA) (Santa Cruz Biotechnology, cat. no. sc-281692)
Methanol (Sigma-Aldrich, cat. no. 322415-2 liters), −20°C
2% normal donkey serum (see recipe) (Sigma-Aldrich, cat. no. D9663-10ML)
Primary antibodies (see Table 1)
Secondary antibodies (see Table 2)
30% sucrose
Tissue-Tek O.C.T. Compound (Sakura, cat. no. 4583)
Hoechst 33342 (Life Technologies, cat. no. H3570)
Fluoroshield™ histology mounting medium (Sigma-Aldrich, cat. no. F6182-20ML)

Table 1 Primary Antibody List

Antibody	Species	Dilution	Company	Catalog number	RRIDs
SOX17	Goat	1:100	R&D Systems	AF1924	AB_355060
FOXA2	Rabbit	1:400	Cell Signaling Technology	3143S	AB_2104878
PDX1	Goat	1:100	R&D Systems	AF2419	AB_355257
NKX6.1	Mouse	1:300	Developmental Studies Hybridoma Bank	F55A10	AB_532378
C-peptide	Rat	1:100	Developmental Studies Hybridoma Bank	GN-ID4	AB_2255626
Glucagon	Guinea pig	1:200	Takara	M182	AB_2619627

Table 2 Secondary Antibody List and DNA Stain

Antibody	Dilution	Company	Catalog number	RRIDs
Goat anti-rat Alexa Fluor® 555	1:500	Life Technologies	A-21434	AB_2535855
Donkey anti-guinea pig Alexa Fluor® 647	1:500	Jackson ImmunoResearch Laboratories	706-605-148	AB_2340476
Donkey anti-goat Alexa Fluor® 555	1:500	Life Technologies	A-21432	AB_2535853
Donkey anti-mouse Alexa Fluor® 488	1:500	Life Technologies	A-21202	AB_141607
Donkey anti-rabbit Alexa Fluor® 488	1:500	Life Technologies	A-21206	AB_2535792
DNA stain Hoechst 33342	1:2000	Life Technologies	H3570	

OLYMPUS 1X73 fluorescent microscope
1.5-ml microcentrifuge tubes
Tissue-Tek cryomolds (Sakura, cat. no. 4566)
Microtome
Superfrost Plus Microscope Slides (Precleaned) (Fisher Scientific, cat. no. 12-550-15)
Dako Pen (Dako, cat. no. s2002)
Slide staining system (Thermo Fisher Scientific, cat. no. c22-038-489)
Humidity chamber
Cover glass (Thermo Fisher Scientific, cat. no. 12-545-J)

Definitive endoderm and pancreatic progenitor stage

Prepare cells for staining

1a. Split cells on Geltrex-coated 4-well plates following Basic Protocol 1.

For each well of 4-well plate, ~0.3 × 10^5 cells are seeded.

2a. Initiate differentiation according to Basic Protocol 2 with 500 µl medium at each differentiation stage.

3a. Aspirate medium from one well of 4-well plate when cells reach day 4 of differentiation.

4a. Rinse one time with PBS by adding 200 µl PBS into the well and then aspirating out the PBS.

5a. Fix cells 10 min by adding 200 µl 4% PFA in well.

6a. Aspirate 4% PFA from the well, rinse one time with PBS, and store at 4°C with 500 µl PBS.

7a. Continue to differentiate well in another 4-well plate to day 12 as described in Basic Protocol 2, and fix cells according to steps 3a through 6a.

Stain cells for specific markers

8a. Aspirate PBS from the well, add 200 µl cold methanol (−20°C) into each well, and place plate 10 min at −20°C.

9a. Aspirate cold methanol, and rinse wells three times with PBS.

10a. Block non-specific staining by adding 200 μl of 2% normal donkey serum into each well and incubate 1 hr at room temperature.

11a. Dilute primary antibodies in 200 μl PBS for each well at a ratio described in Reagents and Solutions.

For definitive endoderm stage well, 2 μl SOX17 and 0.5 μl FOXA2 primary antibodies are diluted in 200 μl PBS.

For pancreatic progenitor stage well, 2 μl PDX1 and 0.6 μl NKX6.1 primary antibodies are prepared in 200 μl PBS.

12a. Incubate cells with primary antibodies overnight at 4°C.

13a. Wash well three times with PBS, and leave the last wash of PBS in the well.

14a. Prepare diluted secondary antibodies and DNA stain at the ratios described in Reagents and Solutions.

For definitive endoderm stage wells, 0.4 μl of each donkey anti-goat Alexa Fluor® 555, donkey anti-rabbit Alexa Fluor® 488, and diluted Hoechst 33342 are diluted in 200 μl PBS.

For pancreatic progenitor stage well, 0.4 μl of each donkey anti-goat Alexa Fluor® 555, donkey anti-mouse Alexa Fluor® 488, and diluted Hoechst 33342 are diluted in 200 μl PBS.

15a. Aspirate PBS from the well, add secondary antibodies prepared for cells at specific stages in each well, and incubate 45 min at room temperature.

16a. Wash well three times with PBS, and leave the last wash of PBS in the well.

17a. Acquire pictures of stained cells using a fluorescent microscope (Fig. 3A,B).

Pancreatic beta cell stage

Prepare cell clusters for staining

1b. Collect five to ten clusters at the end of differentiation in a 1.5-ml microcentrifuge tube.

2b. Aspirate medium and fix cell clusters with 200 μl of 4% PFA for 15 min at room temperature.

3b. Aspirate PFA and add 1 ml PBS to wash clusters.

4b. Remove PBS, add 200 μl 30% sucrose, and incubate overnight at 4°C to allow clusters to precipitate after dehydration.

5b. Remove clusters and transfer them into a cryomold. Use a pipet to remove extra sucrose in mold, add a drop of O.C.T. medium, and mix the clusters with O.C.T. medium.

6b. Place mold horizontally on dry ice and fill the mold to the top with additional O.C.T. medium, and store mold at −80°C after the mold is frozen on dry ice.

7b. Cryosection the frozen block into 5-μm sections on microscope slides using a microtome, and store slides at −80°C until staining.

Stain pancreatic beta cell clusters

8b. Remove slides from −80°C and leave 10 min at room temperature.

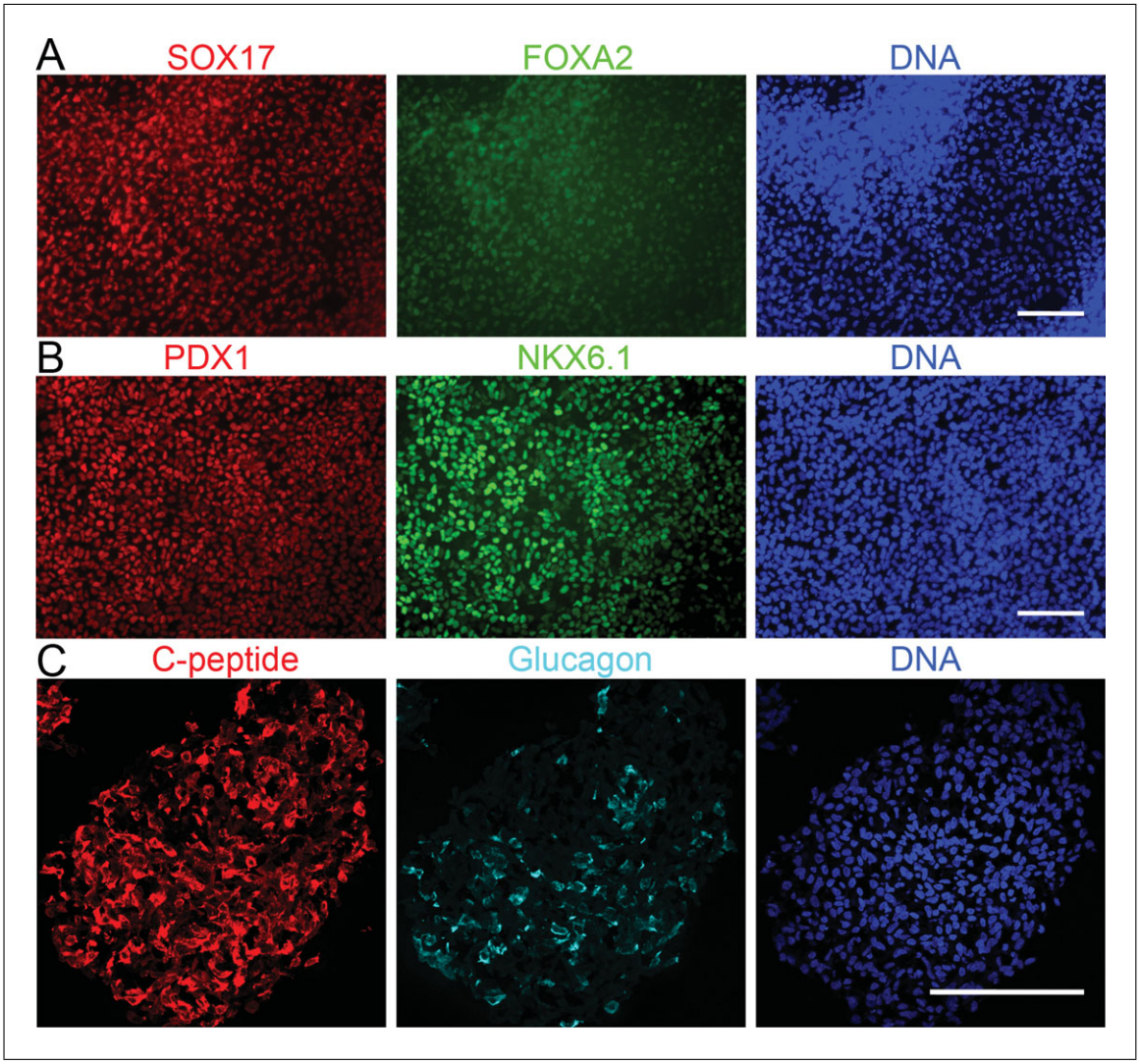

Figure 3 Immunostaining of cells at different stages during differentiation. (**A**) Cells were stained for SOX17 (red) and FOXA2 (green) at definitive endoderm stage. (**B**) Cells were stained for PDX1 (red) and NKX6.1 (green) at pancreatic progenitor stage. (**C**) Beta cell clusters were stained for C-peptide (red) and glucagon (green) at the end of differentiation. DNA stained with Hoechst 33342 in blue. Scale bar: 100 μm.

9b. Circle cell clusters on slides with hydrophobic Dako pen.

This circle provides a barrier to liquids and prevents waste of antibodies solutions.

10b. Rehydrate slides in a slide staining jar containing PBS for 15 min.

11b. Replace PBS with cold methanol, and place jar with slides 10 min at −20°C.

12b. Pour cold methanol back into the bottle at −20°C, wash slides three times with PBS in the jar, and leave the last PBS wash in the jar.

Cold methanol can be reused. Slides should not be dried at any time during the staining.

13b. Remove PBS from the slides, cover slides with drops of 2% normal donkey serum, place slides into a humidity chamber, and incubate 1 hr at room temperature.

14b. Prepare primary antibodies in a 1.5-ml microcentrifuge tube.

For one slide, dilute 1 μl C-peptide and 0.3 μl glucagon antibodies in 100 μl PBS.

15b. Remove donkey serum from slides, add 100 μl diluted antibodies onto each slide, and incubate overnight at 4°C.

16b. Wash slides three times in PBS.

17b. Prepare secondary antibodies and Hoechst 33342 DNA stain.

For one slide, prepare 0.2 μl of each donkey anti-rat Alexa Fluor® 555, donkey anti-guinea pig Alexa Fluor® 647, and diluted Hoechst 33342 in 100 μl PBS.

18b. Remove slides from jar, remove excess PBS, add 100 μl diluted secondary antibodies and DNA stain onto each slide, and incubate 45 min at room temperature.

19b. Wash slides three times with PBS.

20b. Remove excess PBS on slide and add 1 drop of histology mounting medium.

21b. Cover slide with cover glass.

22b. Take pictures of stained slides using fluorescent microscope (Fig. 3C).

REAGENTS AND SOLUTIONS

Cluster medium

DMEM plus GlutaMAX
1% (v/v) penicillin-streptomycin
1% (v/v) B-27 serum-free supplement (50×)
1 μM ALK5 inhibitor (Stemgent, cat. no. 04-0015)
10 μg/ml Heparin (Sigma-Aldrich, cat. no. H3149)
25 ng/ml FGF7
10 μM Y-27632, ROCK inhibitor
Prepare fresh and use up within 3 days stored at 4°C or 1 month at −20°C

Normal donkey serum, 2%

1 ml normal donkey serum (Sigma-Aldrich, cat. no. D9663-10ML)
49 ml D-PBS
Store up to 1 month at 4°C

Pancreatic beta cell stage medium

RPMI plus GlutaMAX
1% (v/v) penicillin-streptomycin
1% (v/v) B-27 serum-free supplement (50×)
10% (v/v) fetal bovine serum (Atlanta Biologicals, cat. no. S11150)
10 μM Y-27632, ROCK inhibitor
Prepare fresh and use up within 3 days stored at 4°C or 1 month at −20°C

Pancreatic endocrine progenitor stage medium

RPMI plus GlutaMAX
1% (v/v) penicillin-streptomycin
1% (v/v) B-27 serum-free supplement (50×)
1 μM thyroid hormone (T3) (Sigma, cat. no. T6397)
10 μM ALK5 inhibitor
10 μM zinc sulfate (Sigma-Aldrich, cat. no. Z4750)
10 μg/ml heparin (Sigma-Aldrich, cat. no. H3149)
100 Nm gamma-secretase inhibitor (DBZ) (EMD Millipore, cat. no. 565789)
10 μM Y-27632, ROCK inhibitor
Prepare fresh and use up within 3 days stored at 4°C or 1 month at −20°C

Pancreatic progenitor stage medium

DMEM plus GlutaMAX
1% (v/v) penicillin-streptomycin
1% (v/v) B-27 serum-free supplement (50×)

50 ng/ml EGF (R&D Systems, cat. no. 236-EG)
25 ng/ml FGF7
Prepare fresh and use up within 3 days stored at 4°C or 1 month at −20°C

PBS

3 PBS tablets (Fisher Scientific, cat. no. BP2944-100)
600 ml distilled water
Store at room temperature

Posterior foregut stage medium

DMEM plus GlutaMAX (Life Technology, cat. no. 10569-044)
1% (v/v) penicillin-streptomycin
1% (v/v) B-27 serum-free supplement (50×)
0.25 μM KAAD-cyclopamine (Stemgent, cat. no. 04-0028)
2 μM retinoic acid (Stemgent, cat. no. 04-0021)
0.25 μM LDN193189 (Stemgent, cat. no. 04-0074)
Prepare fresh and use up within 3 days stored at 4°C or 1 month at −20°C

Primitive gut tube stage medium

RPMI 1640 plus GlutaMAX (Life Technology, cat. no. 61870-127)
1% (v/v) penicillin-streptomycin (Thermo Fisher Scientific, cat. no. 15070-063)
1% (v/v) B-27 serum-free supplement (50×) (Life Technology, cat. no. 17504044)
50 ng/ml FGF7 (R&D Systems, cat. no. 251-KG)
Prepare fresh and use up within 3 days stored at 4°C or 1 month at −20°C

Washing medium

RPMI 1640 plus GlutaMAX (Life Technology, cat. no. 61870-127)
1% (v/v) penicillin-streptomycin
Store up to 2 months at 4°C

COMMENTARY

Background Information

The ability to create insulin-producing cells from human stem cells has greatly expanded experimental options for the study of the molecular physiology of the beta cell in general, and its function in specific pathologic states. Eventually, these cells will very likely be useful in the treatment of diabetes. The fact that these techniques permit creation of beta cells in the context of the cellular complement of a normal islet further enhances their utility for research and, ultimately, therapeutics.

Current protocols—including the one presented here—use a strategy that endeavors to recapitulate *in vitro* the *in vivo* embryological steps in the generation of the pancreas and its endocrine cellular constituents. Activin A and Wnt 3a are effective at inducing definitive endoderm from hESCs *in vitro* (D'Amour et al., 2005). Induction of posterior foregut endoderm can be achieved by the activation of FGF and RA signaling pathways and inhibition of the SHH signaling pathway (D'Amour et al., 2006; Mfopou et al., 2010). Early inhibition of BMP4 signaling enables a shift from hepatocyte lineage to pancreatic lineage (Mfopou et al., 2010). To generate pancreatic progenitors, the protein kinase C (PKC) pathway is activated to induce NKX6.1 before the onset of NGN3 expression (Rezania et al., 2013; Sui et al. 2013). In the embryo, pancreatic endocrine cells develop from Ngn3-positive pancreatic endocrine progenitors. These Ngn3-positive cells are derived from pancreatic progenitors upon downregulation of Notch signaling (Shih et al., 2012). To mimic this stage in vitro, a γ-secretase inhibitor is added to inhibit Notch signaling, resulting in the expression of Ngn3 (D'Amour et al., 2006; Mfopou et al., 2010). To generate monohormonal beta cells in vitro, Pagliuca et al. (2014) as well as Rezania et al. (2014) manipulated additional pathways implicated in the development of the pancreas: TGF beta inhibitor (ALK5 inhibitor) and the BMP4 inhibitor (LDN), thyroid hormone and Notch signaling inhibitor were applied for the generation of C-peptide-positive beta–like cells from

pancreatic progenitors. The protocol presented here was adapted to pluripotent stem cell lines derived in the Egli lab based on these previous studies, and the generation of islet-like clusters with high percentage of C-peptide-positive beta cells from hESCs and iPSCs was demonstrated (Sui et al., 2018). Although these stem cell–derived beta cells appear to be very similar to beta cells of the human pancreas, there are functional differences between stem cell–derived insulin-expressing cells and adult beta cells, including a modest response to glucose, lower insulin content, and reduced insulin processing. Analysis of beta cell function *in vitro* is not recommended, as these cells may not provide an informative result. To avoid teratoma formation, grafting into mice only if >40%, ideally >50%, of cells contained within the cluster express C-peptide is recommended. Functional maturation of islet clusters can be achieved after 2 to 3 months of transplantation in immune-compromised mice. The methods for transplantation are well described (Sui et al., 2018). Upon transplantation, stem cell–derived beta cells form vascularized islet-like structures with MAFA-positive monohormonal C-peptide-positive cells and interspersed glucagon-positive cells. They show a normal proinsulin-to-insulin ratio, and are able to protect mice from diabetes. Future studies will focus on fully functional maturation of stem cell–derived beta cells *in vitro*, from which the modeling of diabetes in a dish will benefit.

Critical Parameters

hPSCs quality and density

The quality and density of hPSCs are critical for efficient differentiation towards beta cells. Use only differentiation-competent stem cell lines that are karyotypically normal and mycoplasma negative. Much time and effort can be lost when performing work with new iPS cell lines that appear pluripotent, but have not sufficiently been quality controlled.

Pancreatic progenitor cells clustering

Cells at this stage are very sensitive to dissociation enzyme. Incubation too long with dissociation enzyme can significantly reduce the number of cells forming clusters.

Troubleshooting

Beta cell differentiation requires a high level of dedication by the researcher and is associated with high costs. Only begin an experiment when fully ready to carry it through carefully to completion and analysis of results. To improve beta cell differentiation, the availability of quantification of the efficiency of differentiation at different stages is key (see Anticipated Results). This will inform where potential problems might be. This protocol is not for blind following, but requires active scientist-culture interactions. Observe cells daily. The most important tool in establishing an efficient protocol is observation.

Low-differentiation efficiency of definitive endoderm at day 4

hPSCs are resistant to differentiation towards definitive endoderm when the cells are too confluent and multiple layers form. These cultures retain Oct4 expression, differentiate poorly, and give rise to teratomas when grafted. To increase the differentiation efficiency, the differentiation should be initiated as soon as possible once hPSCs reach 95% to 100% confluency. Washing the cells before differentiation is also necessary to remove growth factors maintaining pluripotent states in hES medium.

High cell death rate at day 6

High cell death rate can be caused by the persistence of definitive endoderm differentiation factors in the wells and also by using cold medium. To reduce the cell death, wash wells before switching to new medium and warm up the new medium before adding it to wells. Some cell lines are more prone to undergo cell death than others, requiring adaptations in cell density. Choose a differentiation competent stem cell line for the experiment. Among different iPS cell clones from the same cell line, not all will be differentiation competent.

Low-cluster yield

Either a too long or too short dissociation process can cause failure of cluster formation. Monitor cells under a microscope when cells are incubated with dissociation enzyme. Remove enzyme at the proper time point based on the morphology observed under the microscope as described in the protocol.

Clusters merge together into big clusters and have a dense core

Clusters merging into a dense core is caused by culturing too many clusters in each well and failure to evenly shake them before placing them back into the incubator. Big clusters can have a necrotic core due to inaccessibility to the nutrients in the medium. High density of clusters in a well exhausts nutrients quickly and causes cell death (Faleo et al., 2017). An amount of 1000 clusters that are 100 to

200 μm in each well of 6-well plates are ideal for a good shape of clusters. If the clusters are bigger, seed fewer clusters in each well. Shake the plate forwards and backwards and side to side before returning them to the incubator every time when they are taken out for examination and medium change.

Selecting differentiation competent cell lines

Several factors can affect the yield of insulin-expressing cells generated during differentiation. Cell plating density and the experience of the investigator in working with human pluripotent stem cell culture are important factors. However, a key factor of success is the choice of a differentiation competent stem cell line. To learn beta cell differentiation, it is highly advised to use a fully vetted cell line, which can be obtained from the Egli laboratory upon request.

Anticipated Results

For pancreatic lineage competent hPSC lines, 95% SOX17-postive cells are expected at the definitive endoderm stage, 50% to 80% PDX1 and NKX6.1 double-positive cells can be obtained at the pancreatic progenitor stage, and >50% C-peptide-positive cells are expected at the end of differentiation. Among C-peptide-positive cells, ~50% are monohormonal C-peptide-positive cells. Polyhormonal can also be found in differentiated cells.

Time Considerations

Stem cell lines should be cultured for experimentation, and not continually passaged if not needed. Improved cell culture media such as StemFlex allow rapid expansion of pluripotent stem cells upon thawing and should be ready for differentiation within 1 week, depending on the scale. The entire beta cell differentiation process after splitting hPSCs takes 27 days. Analyzing the cells at different differentiation stages may take an additional 5 days for cell fixation and imaging.

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